THE DESIGN AND SYNTHESIS OF POTENTIAL CALMODULIN ANTAGONISTS

by

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Meera B. Thadani

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I ABSTRACT

The binding sites of many major tranquilizers to calmodulin are thought to be located near calcium binding domains I and III. Both sites are part of an amphiphilic α-helix and consist of an aromatic hydrophobic region and an acidic hydrophilic region which may interact with the hydrophobic aromatic center and basic hydrophilic side chains of drugs, respectively.

Using CPK molecular models of the binding site on the N-terminal α-helix of bovine brain calmodulin calcium binding site III, a molecule was designed to produce optimum fit to the predicted drug binding site. The molecules synthesized, E-6-amino-1,2-diphenylhex-1-ene, 6-amino-1,2-diphenylhexane, E-1,2-diphenyl-5-guanidinopent-1-ene and 1,2-diphenyl-5-guanidinopentane consist of two aromatic groups to provide interaction with the two phenylalaninine side chains (Phe 89 and Phe 92) in the hydrophobic region of the binding site and a basic hydrophilic side chain designed to interact with Glu 87 in the hydrophilic region of the predicted drug binding site.

In a preliminary study, E-6-amino-1,2-diphenylhex-1-ene hydrochloride was shown to inhibit calmodulin regulated smooth muscle myosin light chain kinase (MLCK) with an IC_{50} similar to that of chlorpromazine inhibition of calmodulin regulated phosphodiesterase.

II. INTRODUCTION

A. Second Messenger System in Cellular Regulation

The term homeostasis (Greek, homois, homeo-, always the same, unchanging + stasis, standstill) is defined as "a state of physiological equilibrium produced by a balance of functions and chemical composition within an organism."[1] In order to survive an organism must maintain this equilibrium. An imbalance in the intricate network of co-ordinated activity maintaining equilibrium leads to pathological conditions which, if not corrected, may result in the death of the organism.

In a multicellular organism, communication between the cells is a vital part of maintaining homeostasis. In addition to co-ordinating its own functions, the individual cell must work in concert with the other component cells of the organism. To fulfill the need for intercellular communication, each cell possesses cell surface receptors which receive chemical signals and messenger molecules which may generate further signals or relay the chemical signals received. These signals activate or inhibit, via the messenger molecules, the reactions which control the metabolic processes involved in the maintenanace of homeostasis.

Hormones, cyclic 3',5'-adenosine monophosphate (cAMP) and the calcium ion (Ca^{+2}) are some examples of messengers found in mammalian cells[2]. Their activities are intricately interrelated as shown in Fig. 1.[2]

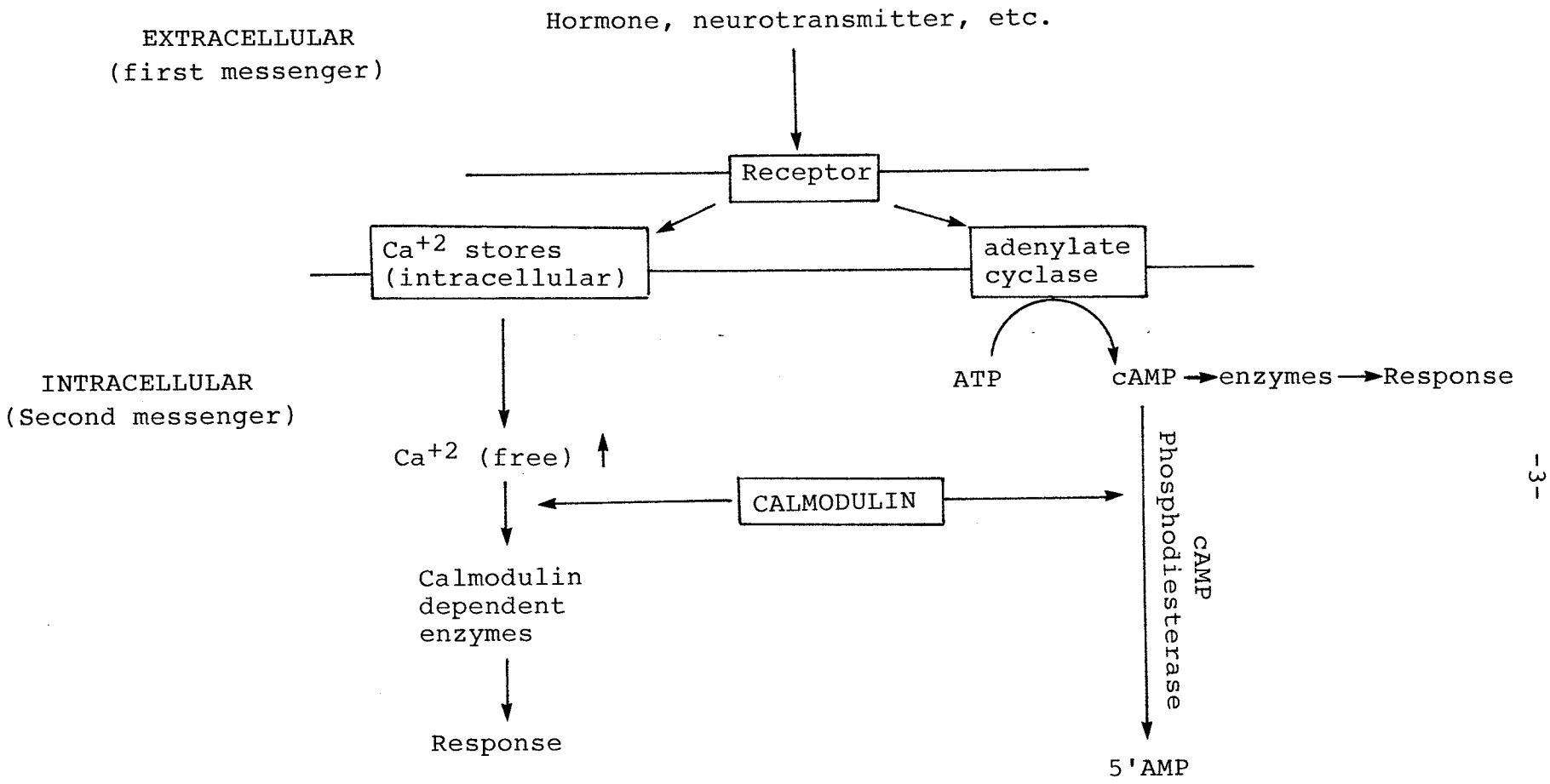


Fig. 1 The figure shows the interplay between the first and second messengers to maintain homeostasis.

The concept of hormones as intercellular chemical messengers dates back to 1905 when William Bayliss and Ernest Henry Starling used the term (hormone, Greek hormon, impulse) to describe the effects of secretin.[1,3] Between 1905 and the 1950's research in endocrinology focused on the isolation identification, analysis and characterization of these chemical messenger molecules. It was not until 1957 that a series of studies to elucidate the mechanism controlling glycogenolysis performed by Sutherland et al. identified cAMP and the enzymes involved in its metabolism (Fig. 2), as the mediators of the effect of the hormone.[4] This led to the concept of the "second messenger" in the control of cellular regulatory mechanisms. The second messenger hypothesis implies that the first messenger, the hormone, regulates the activity of specific cells by regulating the intracellular levels of the second messenger (Fig. 2.)[2]

In the mammalian cell many of the actions of cAMP are in turn mediated by a cAMP dependent protein kinase.[5] The mechanism of action of cAMP on the protein kinase is shown in Fig. 3. The protein kinase is a tetramer composed of two dimers: a dimer receptor subunit (R_2) which binds cAMP and a dimer catalytic subunit (C_2). When cAMP is absent, R_2C_2 is the inhibited enzyme complex. When cAMP binds to R_2, it leads to the dissociation of the R_2C_2 complex. The catalytic subunit C_2, is now free and active.[6] The protein kinases act to phosphorylate cellular proteins and thus co-ordinate cell-

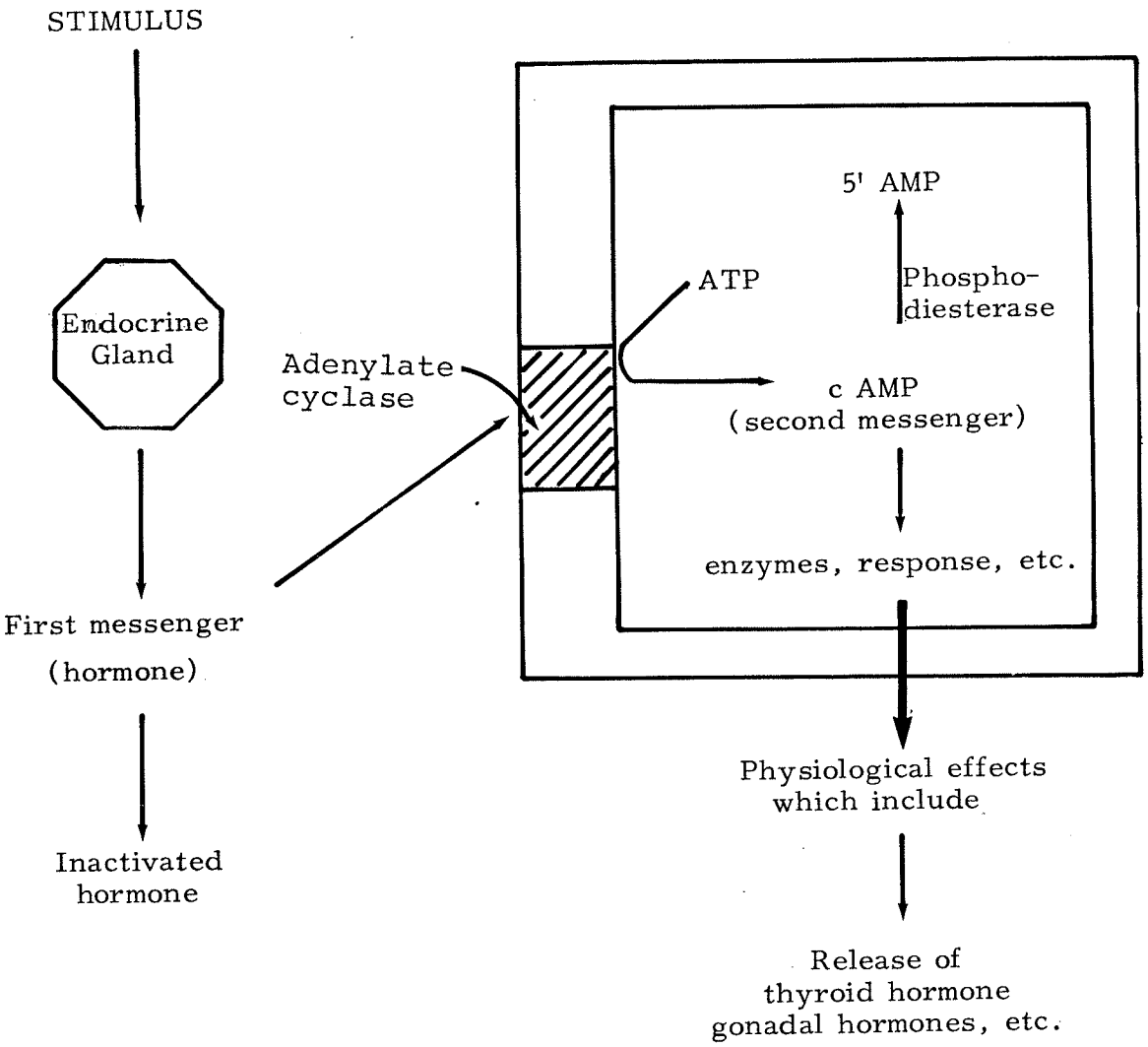
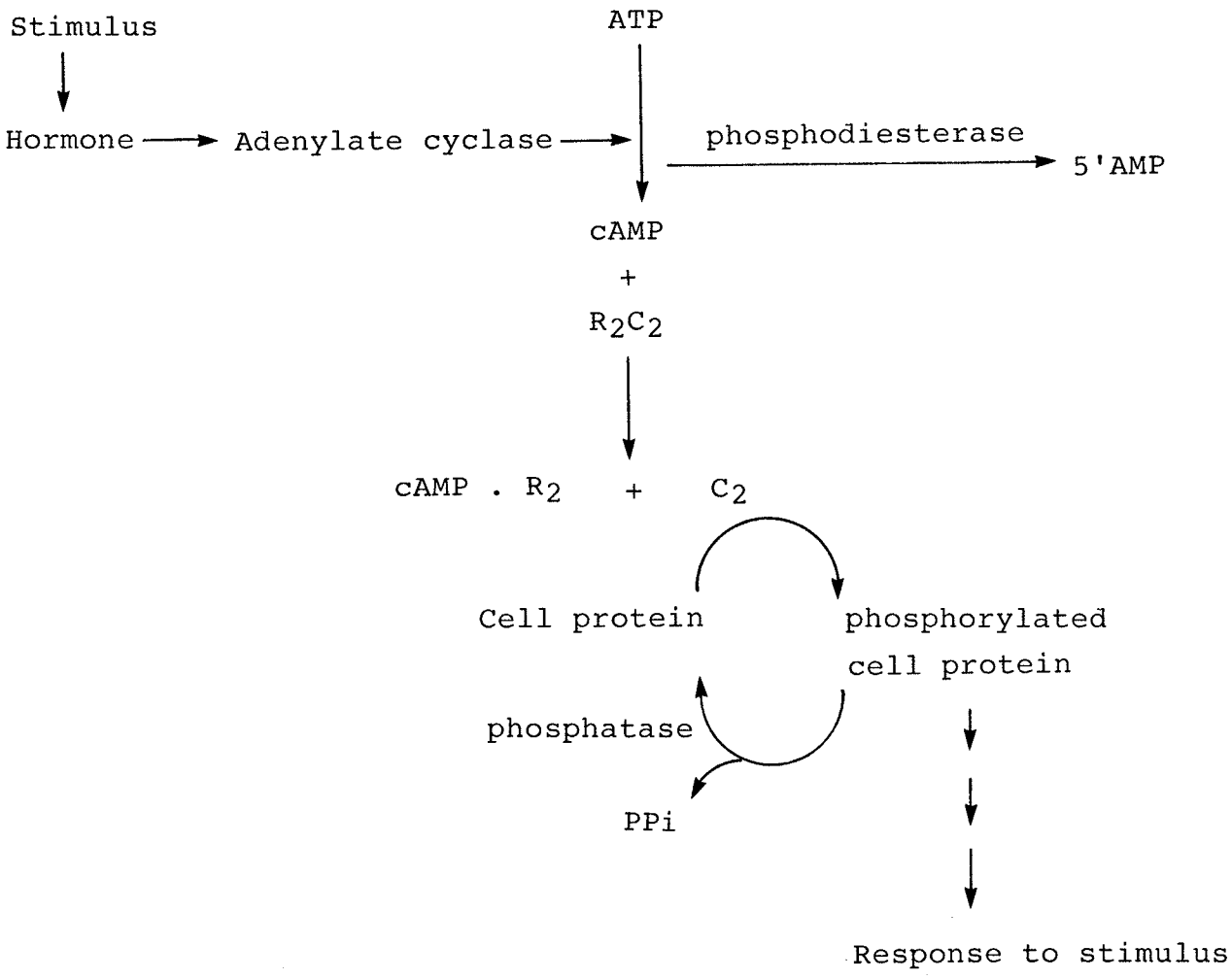


Fig. 2 The second messenger concept of hormone action.

ular activity. The cascade of enzymes involved in glycogenolysis is the best understood model of metabolic regulation by cAMP at the molecular level.[2,5]



R_2C_2 = inhibited protein kinase complex
C = catalytic subunit
R = receptor subunit

Fig. 3 Biological role of cAMP as a second messenger

The role of the calcium ion in the regulation of cellular activity was first observed in 1883 by the English physiologist Ringer.[7] He discovered that if a frog's heart was placed in a calcium free sodium chloride solution it stopped beating. When the heart was examined it was found that it was always in the relaxed state. The readdition of calcium to the sodium chloride solution reactivated the heart to beat regularly. If very high amounts of calcium were added however, the heart again stopped beating and this time it was always in the contracted state. The significance of these findings was not fully realized at that time because the science of cell biology and the technology needed to explore these observations were in their infancy.

It was in 1947 that Heilbrunn and Wiercienski showed that the injection of Ca^{+2} into the muscle fiber causes contraction and proposed that Ca^{+2} was the intracellular messenger involved in this process.[8] Since then research in this area has revealed that the calcium ion is involved in many biological processes and cellular reactions and that calmodulin, a multifunctional Ca^{+2} binding protein is a major receptor of this divalent cation.

The research which led to the discovery of calmodulin did not involve calcium at all. Instead, it focused on the purification and characterization of the enzyme cAMP phosphodiesterase from bovine brain tissue, the object of which was to study the regulatory aspects of cAMP metabolism.[9]

Cheung[9] selected brain tissue because it was readily available and contained high levels of cAMP metabolic enzymes. When crude cAMP phosphodiesterase was passed through an anion exchange column it was found that the activity of the purified enzyme had decreased substantially. This loss of activity was attributed to an "activator" which was removed by chromatography from the crude enzyme preparation.[10] The activator, was purified and shown to stimulate the purified cAMP phosphodiesterase.[11] Having found the activator (i.e. calmodulin), the mode of interaction between calmodulin and cAMP phosphodiesterase became the focus of attention. To find the nature of this interaction, it was necessary to first determine tissue localization (both cellular and sub-cellular) and then purify the protein to permit physical and chemical characterization. Investigation revealed that calmodulin had a broad distribution in eukaryotic tissues implying that besides activating phosphodiesterase, it was involved in other basic cellular functions.[10]

The involvement of Ca^{+2} with calmodulin was demonstrated by Kakiuchi et al.[12,13] in a study which examined the effects of divalent metal ions on cAMP phosphodiesterase. Mammalian phosphodiesterase was known to require Ca^{+2} for activity and it was shown that in the presence of 3mM Mg^{+2} the addition of 10μM Ca^{+2} increased the activity of cAMP phosphodiesterase in brain extracts more than two fold.[12] Furthermore, calmodulin had no effect on cAMP phosphodiesterase

until the threshold level of Ca^{+2} (> 10^{-6}M) was added to the enzyme preparation.[13] Teo and Wang later defined the relationship of Ca^{+2} with calmodulin in a study on cardiac cAMP phosphodiesterase and demonstrated that the Ca^{+2}- calmodulin complex was the activator for cAMP phosphodiesterase.[14]

Since 1973 calmodulin has been found to regulate adenylate cyclase,[15] myosin light chain kinase,[16] plant NAD kinase[17], and has been identified as a subunit of phosphorylase b kinase, the enzyme involved in glycogen degredation.[18] Another important revelation was that calmodulin was structurally homologous to troponin C, the calcium binding protein found in skeletal muscle [19] suggesting that the two share a common evolutionary pathway. Research in this area in the last decade has produced an avalanche of information and insight into the interplay between cAMP and calmodulin in the regulation of many cellular processes. The discovery that calmodulin functions as a mediator for Ca^{+2} and is a regulator of Ca^{+2} dependent adenylate cyclase and phosphodiesterase provides a link at the molecular level between these two intracellular regulators as shown in Fig. 1. As indicated, hormones, the intercellular regulators, provide communication between cells while cAMP and Ca^{+2}, the intracellular regulators, work within the cell to co-ordinate biochemical reactions which result in physiological responses to the stimulus.

Calmodulin had a modest beginning as an unknown activator of cAMP phosphodiesterase before it was established that it was in fact an integrator of two very important intracellular regulators and played a key role in directing cellular activities. The full significance of these findings is only now being determined and elucidation of the exact mode of interaction of Ca^{+2} with calmodulin should provide insight into the molecular mechanism of Ca^{+2} regulation of cellular activity.[20]

B. Distribution and Physiochemical Properties of Calmodulin

Calmodulin is an ubiquitous protein and has been found in all animal species examined.[21] It has also been found in invertebrate species,[22] protozoa,[23] and plants.[24] The concentration varies from one source to another with the highest concentrations being found in the tissues of mammalian brain and testes.[25] Subcellular fractionation studies indicate high amounts in the cytoplasmic fraction and lower amounts in the nuclear, mitochondrial and microsomal fractions.[26] Calmodulin has been purified from many sources and its physiochemical properties are given in Table 1.[27]

Calmodulin is heat resistant but not heat stable having a $t_{\frac{1}{2}}$ of 7 minutes at 100°C. It has a molecular weight of 16,700 which is calculated from its sequence of 148 amino acids. The protein has been sequenced from rabbit skeletal muscle, rat testes, bovine uterus and brain.[28] Comparisons

of these sequences reveal that calmodulin is highly conserved in nature.

The amino acid composition is characterized by the absence of tryptophan and cysteine, a high threonine to serine ratio (12:4), 23 aspartate, 23 glutamate, one histidine and one trimethyl-lysine. The lysine is of particular interest as it helps to identify the protein. The physiological significance of this residue has not been evaluated. The lack of cysteine allows the protein to possess a very flexible tertiary structure. This would enable calmodulin to adopt the various conformations required for the enzymes it regulates. The absence of tryptophan and high phenylalanine-tyrosine ratio give calmodulin a characteristic UV spectrum. The CD cpectrum indicates that the protein has a high α-helical content. The binding of Ca^{+2} to the protein increases helicity. This change in conformation is required for the regulation of calmodulin dependent enzymes.[27]

Sedimentation equilibrium and SDS gel electrophoresis experiments in the presence and absence of Ca^{+2} give molecular weight values between 15,000 and 19,000 (Mr) indicating that calmodulin exists as a monomer. The high ratio of acidic to basic amino acid residues accounts for the low isoelectric point of 3.9.

TABLE 1[27]

General Physiochemical Properties of Mammalian Calmodulin

Physical Property	Value
Calcium binding (Ca^{+2})	4 mole/mole
Conformation	40-55% α-helix
Drug binding	phenothiazines and others
Heat resistance	$t_{\frac{1}{2}}$ = 7 minutes at 100°C
Hydrophobicity	increased by Ca^{+2}
Isoelectric pH	3.9
Molecular weight	16,700 (148 amino acids)
Trimethyl-lysine	lysine 115
Sequence homology	50% to troponin C
SDS gel electophoresis ± Ca^{+2}	15,000-19,000 (Mr)

C. Structure of Calmodulin

The amino acid sequence of calmodulin is shown in Fig. 4 and appears to be well conserved in nature.[28] In addition, more than 70% of the amino acid sequence in calmodulin shows homology to those of other Ca^{+2} binding proteins troponin C and parvalbumin, in spite of the fact that the three proteins are functionally distinct.[30] Another interesting feature observed in the primary sequence of calmodulin is its internal homology. Calmodulin can be sub-divided into four calcium binding domains. A comparison of the four domains indicates that domain I (residues 8-40) is homologous to domain III (residues 81-113) and domain II (residues 44-76) is homologous to domain IV (residues 117-148).[21] Based on the crystal structure of parvalbumin[31] and its amino acid homology to troponin C and calmodulin, the calcium binding residues were identified as shown in Fig. 4. Each calcium binding domain has a 12 amino acid loop flanked on each side by an eight to ten amino acid residue α-helix which creates a cavity to harbour the calcium ion.[32]

Attempts to relate structure to calmodulin regulatory activity reveal that the biological activity of the molecule depends upon the integrity of the protein. Controlled tryptic digestion of calmodulin produced fragments which were essentially inactive in stimulating phosphodiesterase.[33] Peptide 1 (residues 1-77) and peptide 2 (residues 78-128) showed no activity. Peptide 3 (residues 1-106) was found to be only

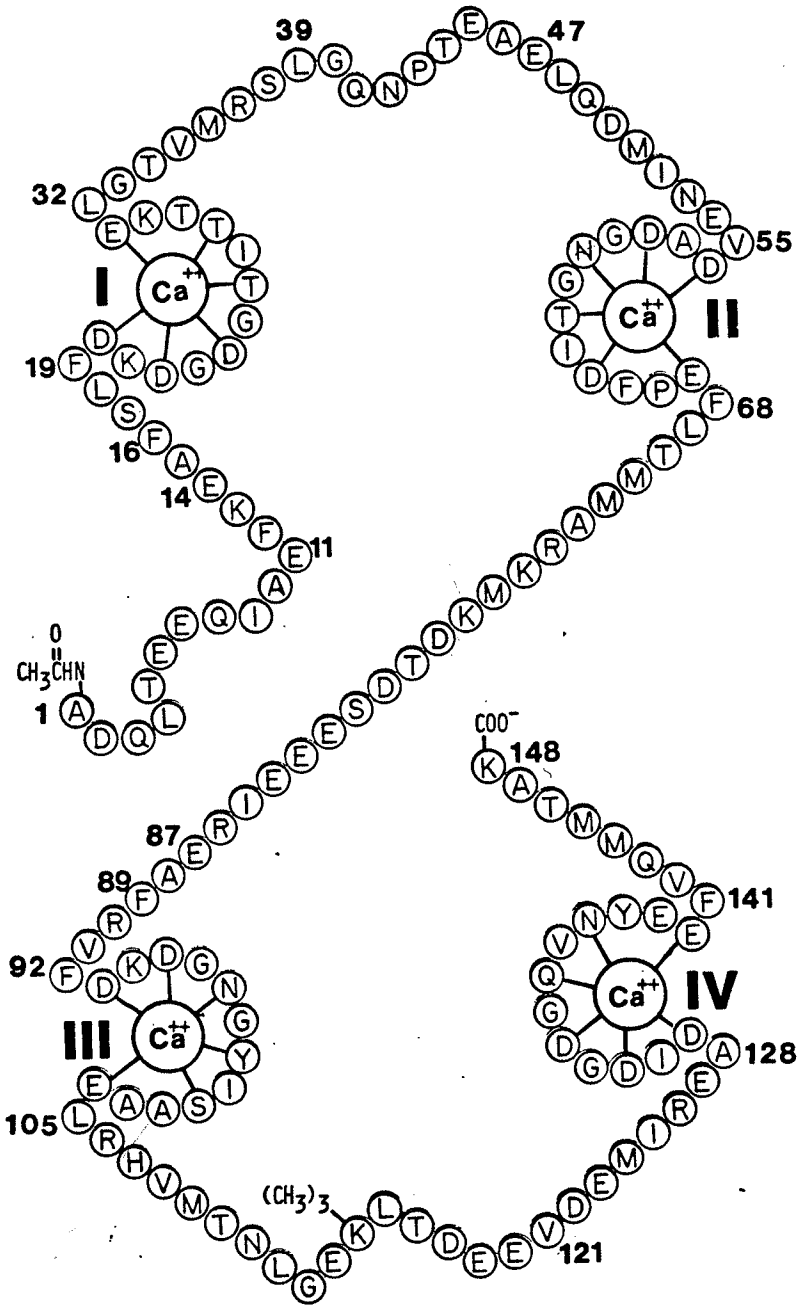


Fig. 4. Amino acid sequence of bovine brain calmodulin.[28] Amino acids are designated by their one letter codes.[29] Amino acids co-ordinating the calcium ion are indicated by solid lines.

1/200th as active as the native sequence, and the removal of residues 91-106 from peptide 3 left an inactive fragment. Chemical modification of one or more methionine or lysine residues or blockade of several carboxyl groups resulted in partial or total loss of activity.[21,34] It appears then that the role of calmodulin is of such fundamental importance in cellular function that a mutation resulting in a gross change in its primary sequence would be lethal to the cell. Indeed, the observation that the primary sequence is highly conserved between phylogenetically distant species lends strong support to this suggestion.[21]

D. Ca^{+2} Binding to Calmodulin

The involvement of Ca^{+2} with calmodulin was first demonstrated by Kakiuchi et al.[11,12] Although some controversy still exists with regards to the relative affinities of each of the four binding sites for Ca^{+2}, binding studies indicate that the protein binds four Ca^{+2} per mole with high affinity.[34] Upon binding Ca^{+2}, the protein undergoes a significant conformational change as measured by circular dichroism and optical rotatory dispersion.[27] The α-helical content of calmodulin increases from about 40% in the absence of Ca^{+2} to greater than 50% in the presence of Ca^{+2}. It is this conformational change which allows calmodulin to interact with its target enzymes and pharmacological agents such as the phenothiazines.[27] The question arises as to how the protein can

regulate so many different enzymes. It is possible that the regulation of these molecules is a function of the number of Ca^{+2} ions bound to calmodulin and the conformational changes which accompany Ca^{+2} binding. The order in which these sites are filled and the discrete conformational changes which result from this, are still the subject of ongoing research.[36]

The three dimensional structure of calmodulin has been determined crystallographically in the presence of calcium by Babu et al. and indicates that the molecule is shaped like a dumbbell.[37] The two lobes of the dumbbell contain two Ca^{+2} binding sites each and are connected to each other by an eight turn α-helix. Each calcium binding site is characterized by a helix-loop-helix (Fig. 4) arrangement which coordinates the Ca^{+2} ion within the loop. The amino acids involved in this interaction for all four Ca^{+2} ions are indicated in Fig. 4. The molecule is stabilized by multiple interactions between the helices as well as hydrogen bonding between adjacent Ca^{+2} binding loops in each half of the molecule.[37]

The eight turn α-helix between Ca^{+2} binding domains II and III (residues 68-92) is of particular interest because evidence suggests that the residues in this helix play an important role in the interaction of calmodulin with target proteins and drugs.[38,39] Proteolysis studies of calmodulin show that in the presence of Ca^{+2}, limited proteolysis produ-

ces two fragments comprising residues 1-77 and 78-148 while in the absence of Ca^{+2}, fragments consisting of residues 1-106, 1-90 and 107-148 are produced. This indicates that calcium binding exposes the residues in the eight turn α-helix to proteolysis.[37] Crystallographic studies on troponin C and calmodulin also indicate the presence of an eight turn α-helix.[37]

E. Mechanism of Enzyme Activation by Calmodulin

The mechanism of action of calmodulin was first determined in the calmodulin-dependent phosphodiesterase system (Fig. 2).[12,14,35] Calmodulin by itself is not active.[12,14] The binding of Ca^{+2} to calmodulin induces a change in its conformation and it is this Ca^{+2}-calmodulin complex which is now active.[33] The active complex then binds to the enzyme phosphodiesterase producing a Ca^{+2}-calmodulin-phosphodiesterase complex which catalyzes the breakdown of cAMP to 5'AMP (Figs. 1,5). This two step activation process, as indicated in Fig. 5 is reversible.

According to this mechanism, the cellular flux of Ca^{+2} plays a key role in regulating phosphodiesterase activity. In mammalian cells the concentration of Ca^{+2} in the cytosol is 10^{-8} to 10^{-7}M. An increase in Ca^{+2} to 10^{-6} or higher is sufficient to allow the formation of the Ca^{+2}-calmodulin complex which can combine with the target enzyme to trigger the biochemical reaction which culminates in a physiological response. The binding of calcium to calmodulin induces conforma-

tional changes creating a hydrophobic surface on the protein which may be the site of interaction of calmodulin with its target enzymes and drugs.[21,25]

1. $\text{Calmodulin}_{(\text{inactive})} + n\text{Ca}^{+2} \rightleftharpoons \text{Calmodulin}^{*}\text{-}n\text{Ca}^{+2}{}_{(\text{active})}$

2. $\text{Calmodulin}^{*}\text{-}n\text{Ca}^{+2}{}_{(\text{active})} + \text{E}_{(\text{less active})} \rightleftharpoons \text{E}^{*}\text{-Calmodulin}^{*}\text{-}n\text{Ca}^{+2}{}_{(\text{active})}$

n = number of Ca^{+2} bound to calmodulin; n = 1 to 4

E = enzyme regulated by calmodulin

* = a new conformation

Fig. 5 Enzyme regulation by calmodulin

Activation of the enzyme by the Ca^{+2}-calmodulin complex is due to conformational changes induced in the enzyme as the Ca^{+2}-calmodulin-enzyme complex is formed.[25] Dissociation of the active enzyme complex results from lowering cellular Ca^{+2}. This decrease of cellular Ca^{+2} results in the dissociation of Ca^{+2} from the complex which causes a change in the conformation of calmodulin to its inactive form. The inactive form of calmodulin dissociates from the enzyme returning enzyme activity to its steady state level.[40] An alternative mode of deactivation involves dissociation of the Ca^{+2}-calmodulin moiety from the Ca^{+2}-calmodulin-enzyme complex followed by inactivation of calmodulin by dissociation of calcium.

Other enzymes which have been found to be under the control of calmodulin include adenylate cyclase, myosin light chain kinase, phosphorylase b kinase, glycogen synthetase kinase and NAD^+ kinase.[25,41] With the exception of phosphorylase b kinase, which has calmodulin as a nondissociable integral component,[42] the enzymes regulated by calmodulin appear to be modulated as shown in Fig. 5.[25]

F. Drug Binding to Calmodulin

The discovery that drugs could alter the activity of calmodulin resulted from studies which attempted to explain the influence of the major tranquilizers on cAMP metabolism. Data showed that these agents inhibited calmodulin regulated phosphodiesterase (Fig. 1) in a Ca^{+2} dependent manner and that this inhibition was related to the clinical effectiveness of these drugs.[43] It was further found that this inhibition was caused by the drug binding to calmodulin rather than to phosphodiesterase.[44,45] That is, the drug was binding to calmodulin, and interfering with its interaction with the enzyme thereby altering enzyme activity. The significance of these findings is that the inhibition of calmodulin may be the basis for explaining the antipsychotic activity of these drugs. Two lines of evidence support this suggestion. First, a number of clinically effective drugs exhibiting antipsychotic activity bind to calmodulin in a Ca^{+2} dependent manner while other centrally acting agents devoid of anti-

psychotic activity do not.[26] Second, it is known that calmodlin regulates the enzymes involved in cyclic nucleotide metabolism (Fig. 1). These nucleotides mediate the actions of the catecholaminergic neurotransmitters which are believed to play a role in diseases, (such as schizophrenia) of the CNS.[43,44,45] Inhibition of calmodulin would affect this process and may explain the pharmacological actions of these therapeutic agents.

The drug binding property of calmodulin can be exploited to reveal the role it plays in regulating biochemical processes. Because the major tranquilizers bind calmodulin, and in the process inhibit its interaction with target enzymes, it can be suggested that the drug/calmodulin interaction has some similarity to the calmodulin/protein interaction.[38] Localizing the drug binding site can lead to an understanding of the drug interaction at the molecular level as well as the calmodulin/protein interaction itself. Furthermore, this information can lead to the design of molecules targeted to "fit" the drug binding site and thereby produce clinically effective compounds.

Fragments of calmodulin consisting of residues 1-77 and 78-124 have been isolated and demonstrated to bind to a fluphenazine-Sepharose column in the presence of Ca^{+2}.[46] This indicates that in addition to the two Ca^{+2} binding sites, these regions also possess at least one drug binding site. The region, consisting of residues 80-113 of the fragment

comprising residues 78-124 of calmodulin, is homologous to residues 90-123 of rabbit skeletal troponin C and drug binding studies with a synthetic peptide corresponding to this region indicate that several clinically active major tranquilizers do interact in this region.[38,47] This has led to the suggestion that one of the binding sites on calmodulin resides between residues 80-113.

Based on the nature of the postulated drug binding site and structures of the drugs known to bind to it, a series of compounds were designed to "fit" this region. This thesis describes the design and synthesis of potential calmodulin antagonists based on the hypothetical drug binding site located near calcium binding domain III.

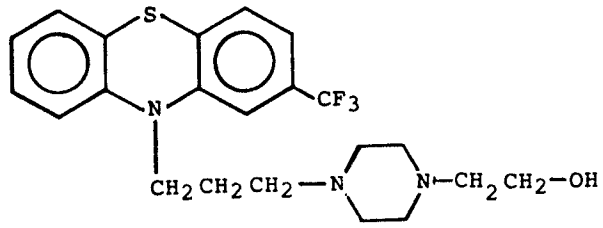
III. DESIGN

A. Drug Interaction With Calmodulin

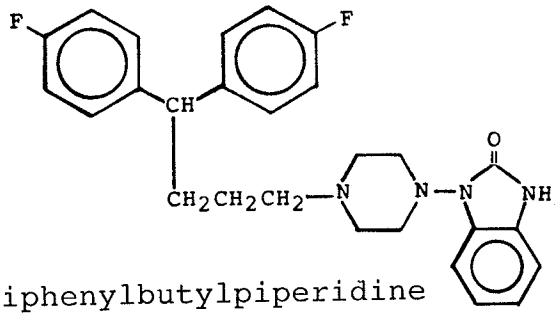
Structural derivatives of the phenothiazines (fluphenazine), diphenylbutylpiperidines (pimozide), butyrophenones (benperidol) and structurally related phenylpiperidines (R-6033) have been pharmacologically classified as major tranquilizers (Fig. 6) These drugs are used in the treatment of psychoses which include schizophrenia, organic psychoses and the manic phase of manic depressive illness. They are also occasionally used to treat depression or severe anxiety.[48]

In man, these drugs cause drowsiness, slow responses to external stimuli, disinterest and lack of initiative. The subjects are easily aroused however, and are capable of giving appropriate answers to direct questions. Intellectual ability, therefore, does not seem to be affected. Psychotic patients become less aggressive, impulsive, agitated and restless. Psychotic symptoms of hallucinations, incoherent thinking and delusions disappear gradually over a period of days.[48]

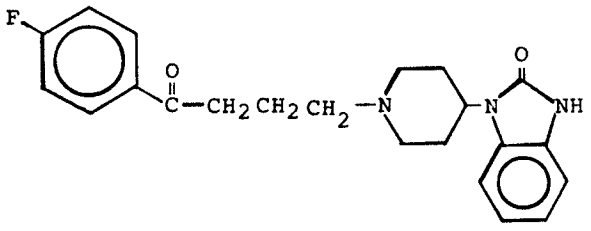
Side effects associated with the use of antipsychotic drugs include rigidity and tremors similar to those observed in Parkinsonism as well as akathisia. Other problems include dystonic reactions (facial grimacing) and tardive dyskinesia (involuntary facial movements) which can be treated by the administration of anticholinergic antiparkinsonism drugs such



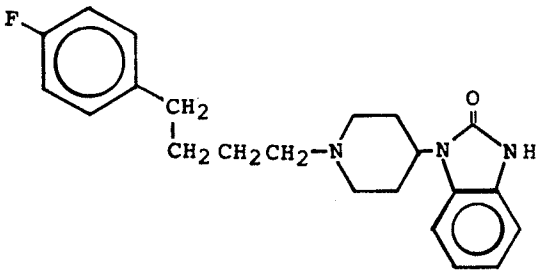
(a) Phenothiazine (fluphenazine)



(b) Diphenylbutylpiperidine (pimozide)



(c) Butyrophenone (benperidol)



(d) Phenylbutylpiperidine (R-6033)

Fig. 6 Structural formulae of some major tranquilizers

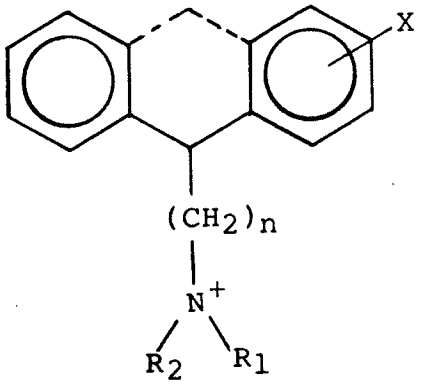
as procyclidine (Kemadrin™).[48]

The mechanism of action of these drugs as major tranquilizers has as yet to be defined at the molecular level. It is currently believed than an imbalance between cholinergic and dopaminergic nerve impulse transmission in the brain is responsible for the complex neurological disorders known as schizophrenia and Parkinsonism.[49] It is known also that the second messenger cAMP is involved as a mediator for these neurotransmitters.[49] The discovery that major tranquilizers inhibit calmodulin dependent cAMP phosphodiesterase in brain tissue by binding to calmodulin led to the speculation that calmodulin may be the receptor site for these drugs.[45] Because calmodulin regulates other enzymes as well, the binding of these drugs to the protein lends support to the suggestion that the many pharmacological effects of these drugs may be explained by a common mechanism of binding to, and inhibiting calmodulin.

B. Structure Activity Relationships of Calmodulin Inhibitors

Examination of the chemical structures of the major tranquilizers known to bind calmodulin in a calcium dependent manner reveals that they have many structural similarities (Fig. 6). All possess an aromatic hydrophobic region separated from a basic center by an alkyl chain of 3 or 4 carbon atoms in length. The basic group carries a positive charge at neutral pH giving the structure amphiphilic character.

In terms of potency, the diphenylbutylpiperidines (eg. pimozide) containing two aromatic rings connected by a single carbon atom are the most effective inhibitors. These are followed by the phenothiazines (eg. fluphenazine) which are tricyclic systems consisting of two aromatic rings and a heterocyclic system. The butyrophenones (eg. benperidol) and phenylbutylpiperidine (R-6033) contain one aromatic ring and are less potent for inhibiting calmodulin sensitive phosphodiesterase.[50,51] Within the phenothiazine group, ring substitutions increasing hydrophobicity of the aromatic region lead to more potent analogues.[50,51] From these observations a generalized chemical structure of calmodulin antagonists can be drawn as shown in Fig. 7.



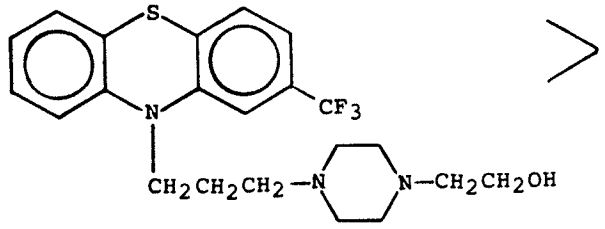
n = at least 3 carbon atoms

X = substituent increasing hydrophobicity of the aromatic system; CF_3 > F > Cl > H > CH_3

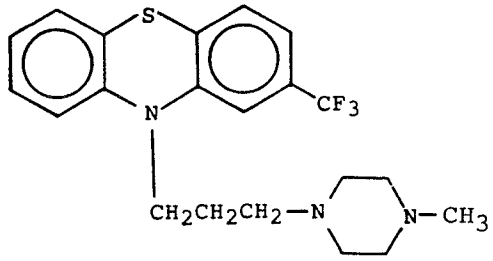
Fig. 7 General structure of calmodulin antagonists.[51]

Given the structures of the drugs which bind to calmodulin and their amphipathic nature, attempts have been made to evaluate structure-activity relationships. Studies relating calmodulin inhibition of phosphodiesterase with hydrophobicity indicate a good correlation between the two.[44] This indicates that the hydrophobic nature of the aromatic nucleus may be an important factor in the drug/protein interaction. This is further supported by the demonstration that the binding of calcium to calmodulin exposes hydrophobic regions which may interact with these drugs.[52,53,54] Additional support for a drug binding site comes from the purification of a calmodulin fragment consisting of residues 78-124 on a fluphenazine-Sepharose column[46] indicating the presence of a phenothiazine interacting site in this region.

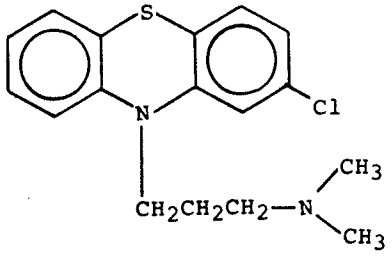
There is no direct correlation between the substituents on the amino group and inhibition of calmodulin function. The position of the amino group however, seems to be important for inhibitory activity because the most potent inhibitors of calmodulin (pimozide and fluphenazine) have a charged amino group three carbon atoms removed from the bridge atom between the aromatic rings (Fig. 7). Compounds in which the chain is less than three carbon atoms in length (eg. promethazine, Fig. 8) appear less potent. Phenothiazines with chain lengths of more than four carbon atoms have not been examined.[55,56] A phenothiazine with an acidic side chain [3-(2-chloro-10-phenothiazinyl) propionic acid] exhibited no anticalmodulin activity.[55]



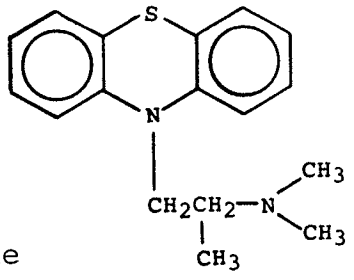
(a) Fluphenazine



(b) Trifluoperazine



(c) Chlorpromazine



(d) Promethazine

Fig. 8 Relative inhibitory activity of the phenothiazines.

C. The Binding Site

Evidence for the presence of a drug binding site on the C-terminal half (residues 78-148) of calmodulin came from Head et al.[46] who were able to isolate a fragment of calmodulin (residues 78-124) and demonstrate calcium sensitive binding to a fluphenazine-Sepharose column. Reid et al. demonstrated calcium sensitivity of a synthetic 34 residue peptide fragment (residues 90-123) corresponding to calcium binding site III of rabbit skeletal troponin C. In spite of the fact that the amino acid sequence of the synthetic peptide analog of rabbit skeletal troponin C is not identical to the corresponding region of bovine brain calmodulin (residues 80-113), similarities do exist as a result of the sequence homology of calcium binding proteins as shown in Fig. 9.[38,47]

Preliminary qualitative CD studies using drugs belonging to the phenothiazine groups indicated interaction with the synthetic peptide.[38] The addition of these drugs to the synthetic peptide in the absence of Ca^{+2} induced α-helical structure as detected by CD. This interaction did vary with the structure and correlated with the antipsychotic activity of the phenothiazines; that is, fluphenazine > trifluoperazine > chlorpromazine > promethazine. The addition of Ca^{+2} to the drug/peptide mixture induced a further α-helical structure in the peptide indicating that Ca^{+2} sensitivity was retained in the presence of the drug. The butyrophenones did not indicate any binding similar to the phenothiazines with the

peptide. The calcium sensitivity of the peptide was maintained in the presence of the butyrophenones (Fig. 10).

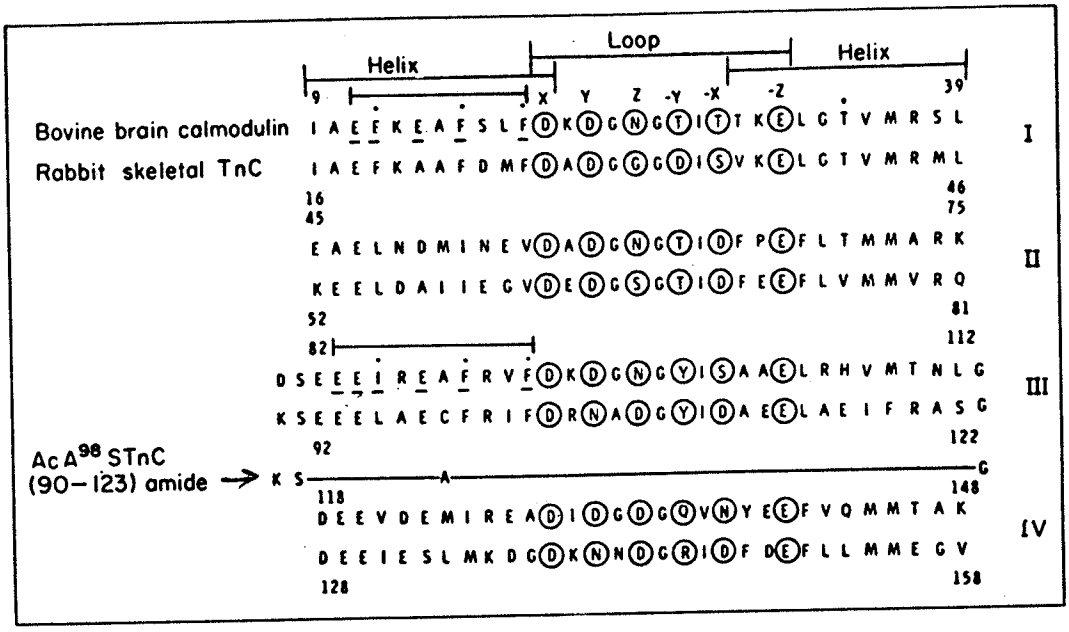


Fig. 9 Amino acid sequence of the calcium binding sites in bovine brain calmodulin, rabbit skeletal troponin C and a synthetic 34-residue calcium binding peptide AcA^{98}STnC (90-123) amide. The calcium binding sites are numbered I-IV. Bovine brain calmodulin is the upper sequence while rabbit skeletal troponin C is the lower sequence. The sequence of the 34-residue peptide [AcA^{98}STnC(90-123)amide] is indicated directly below rabbit skeletal troponin C site III and has an identical sequence to this site with the exceptions indicated. The sequences comprising the helix-loop-helix regions are indicated by bars at the top of the figure. The six octahedral Ca^{+2} coordinating positions in the loop region are designated X, Y, Z, -Y, -X, -Z. The amino acid residues in the corresponding Ca^{+2} coordinating positions are circled. The postulated drug binding sites are indicated by bars over the N-terminal helix regions of calcium binding sites I and III. The residues with side chains postulated to be involved in drug binding are underscored in each site. The residues indicated by an asterisk are those thought to be involved in the hydrophobic core of the protein based on sequence homology of these proteins with parvalbumin, the crystal structure of which is known. Amino acid residues are designated using the one letter code.[38]

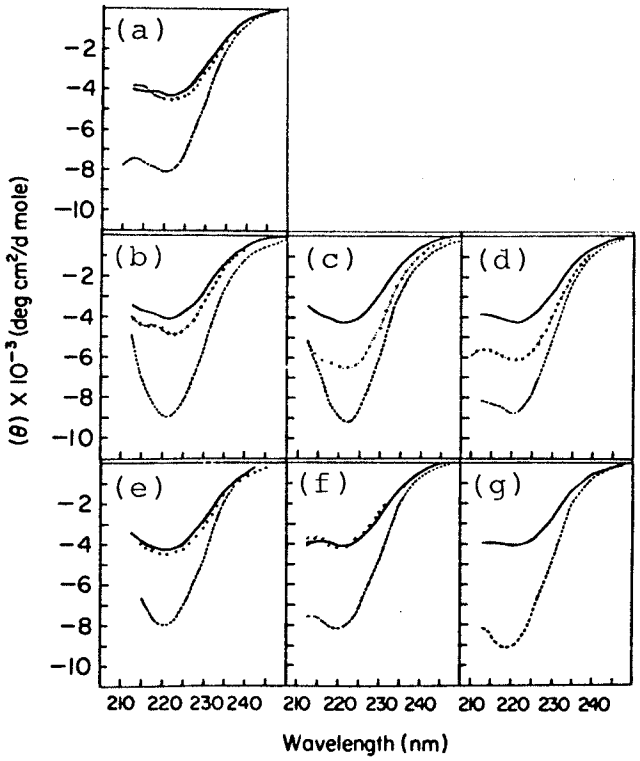


Fig. 10 Drug and calcium induced circular dichroism spectra of the 34 residue peptide, AcA^{98}STnC(90-123) amide. Native peptide (——) peptide and drug (....), peptide and drug and Ca^{+2} (---). The peptide concentration is 0.43 mM. The drug/peptide ratio is 2.3/1. The drugs used are as follows: (a) promethazine; (b) chlorpromazine; (c) fluphenazine; (d) trifluoperazine; (e) benperidol; (f) haloperidol; (g) the circular dichroism spectra of the peptide in the presence (---) and absence (——) of calcium.[38]

These results supported those of Levin and Weiss[45,48] which demonstrated that the phenothiazines were better inhibitors of calmodulin function than the butyrophenones. The peptide studies showed that the binding of phenothiazines was independent of calcium concentration. This was contrary to the observation that the binding of these drugs is calcium dependent in calmodulin.[38] A possible explanation for this is that the 34 residue fragment is more flexible than the entire protein and can therefore interact with the drugs more readily.[37] These observations indicate that the synthetic peptide may have a site of interaction with the major tranquilizers and that this site is similar in structure to the drug binding site in calmodulin.

The amino acid sequence of the synthetic peptide homologous to the calcium binding site III on calmodulin (residues 80-113 , Fig. 9) possesses a helix-loop-helix arrangement in the presence of calcium. A CPK model corresponding to residues 82-93 of calmodulin reveals that these residues may form an amphiphilic α-helix as shown diagrammatically in Fig. 11. Hydrophobic amino acid residues (Ile 85, Phe 89 and Phe 92) are separated from the charged hydrophilic residues (Glu 83, Glu 84 and Glu 87) by one half turn of the α-helix. Therefore it is possible that the hydrophobic region of the phenothiazines interacts with the hydrophobic region of the protein followed (or preceded) by an interaction of the basic side chain of the drug with the acidic hydrophilic

region of the protein. The alkyl chain must be of an appropriate length to allow maximum interaction between the hydrophobic and basic hydrophilic ends of the drug with the corresponding regions on the amphiphilic α-helix on the protein.[38,39] A recent theoretical study by Gresh and Pullman[57] using computer analysis of the intra and intermolecular interactions of the binding of phenothiazine derivatives to residues 82-93 is in agreement with the amphiphilic α-helix hypothesis originally proposed by Reid.[38,39]

Keeping in mind the structure of the drugs known to bind calmodulin and their amphiphilic character, and the amphiphilic nature of the binding site located near calcium binding domain III, a molecular structure was designed to "best fit" this site. Aided by CPK molecular models, it was proposed that the compound in Fig. 12(a) would interact with the amphiphilic α-helix on calmodulin in a fashion similar to the phenothiazine/calmodulin interaction shown in Fig. 11. This compound consisted of two unsubstituted aromatic groups oriented trans to each other by a double bond to provide maximum interaction with Phe 89 and Phe 92 in the hydrophobic region of the binding site. This hydrophobic end of the molecule was to be separated from a basic amino function by an alkyl chain of three or four carbon atoms. The alkyl chain would permit the amino function to extend towards and interact with Glu 87 in the hydrophilic region of the binding site. The double bond in the compound could

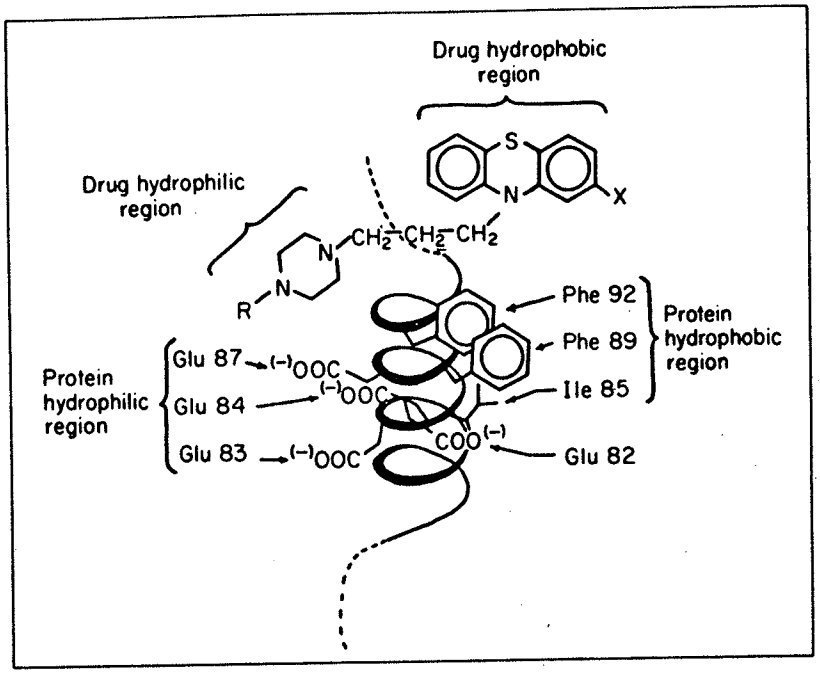
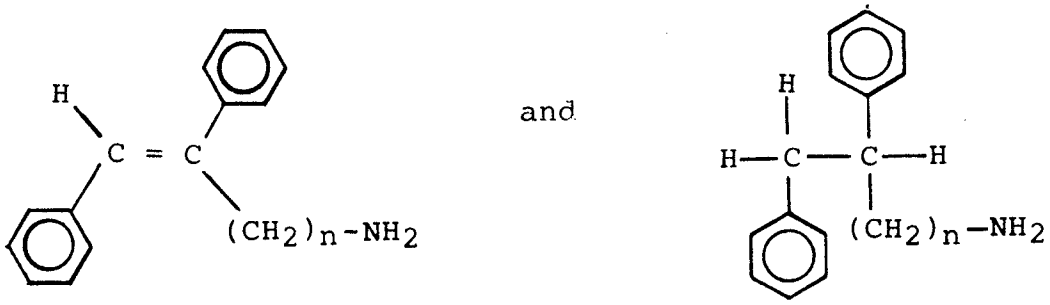


Fig. 11 Schematic representation of the hypothetical drug binding site. The side chains of the key residues Phe 92, Phe 89, Glu 87, Ile 85, glu 84, Glu 83 and Glu 82 are shown extending from the α-helix backbone. the hydrophobic region in the protein consisting of the Phe 92, Phe 89 and Ile 85 is predicted to interact with the tricyclic, hydrophobic region of the phenothiazine tranquilizers. The drug hydrophobic region is separated from the drug hydrophilic region by an alkyl chain "spacer". The spacer is sufficiently long enough (minimum three carbon atoms) to allow the positively charged hydrophilic center in the drug to interact with the negatively charged acidic hydrophilic center in the protein (consisting of Glu 83, Glu 84 and Glu 87) simultaneous with the hydrophobic interaction.[38]

be saturated to give a molecule in which the rings were no longer held in a fixed configuration allowing evaluation of flexibility of the ring in this aromatic region (Fig. 12).



E - isomer (a)

n = 3 or 4 (b)

Fig. 12 General structure of potential calmodulin antagonists bearing an amino group on the side chain.

Similarly, a pair of compounds possessing a guanidino substituted side chain (Fig. 13) would be prepared to see whether the more basic guanidino group has any effect on the inhibitory activity of the molecule. The guanidino group

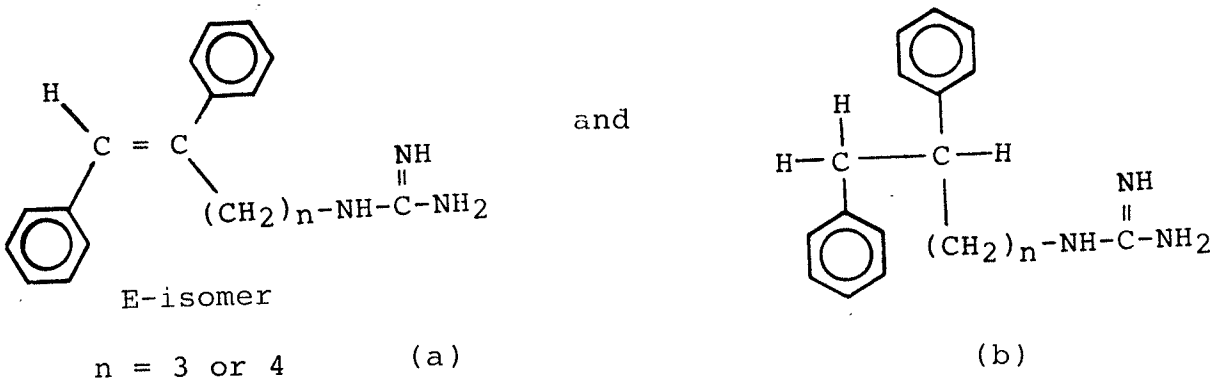


Fig. 13 General structure of potential calmodulin antagonists bearing a guanidino group of the side chain.

is capable of forming ionic linkages with carboxylate groups as shown in Fig. 14.[53] This interaction would tend to favour the binding of compounds bearing the guanidino group to the binding site on calmodulin because the guanidino group can form an ionic linkage with Glu 87.

The compounds synthesized would then be tested for their inhibitory activity in the calmodulin regulated myosin light chain kinase enzyme system to see whether the results supported the amphiphilic α-helix hypothesis.

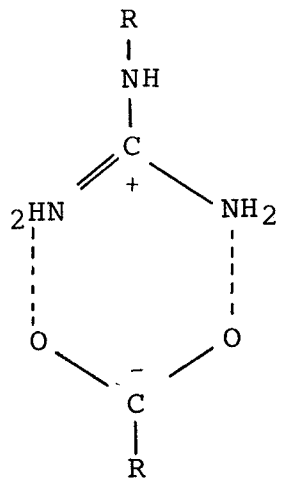


Fig. 14 Ionic interaction between the guanidino group and carboxylate group.

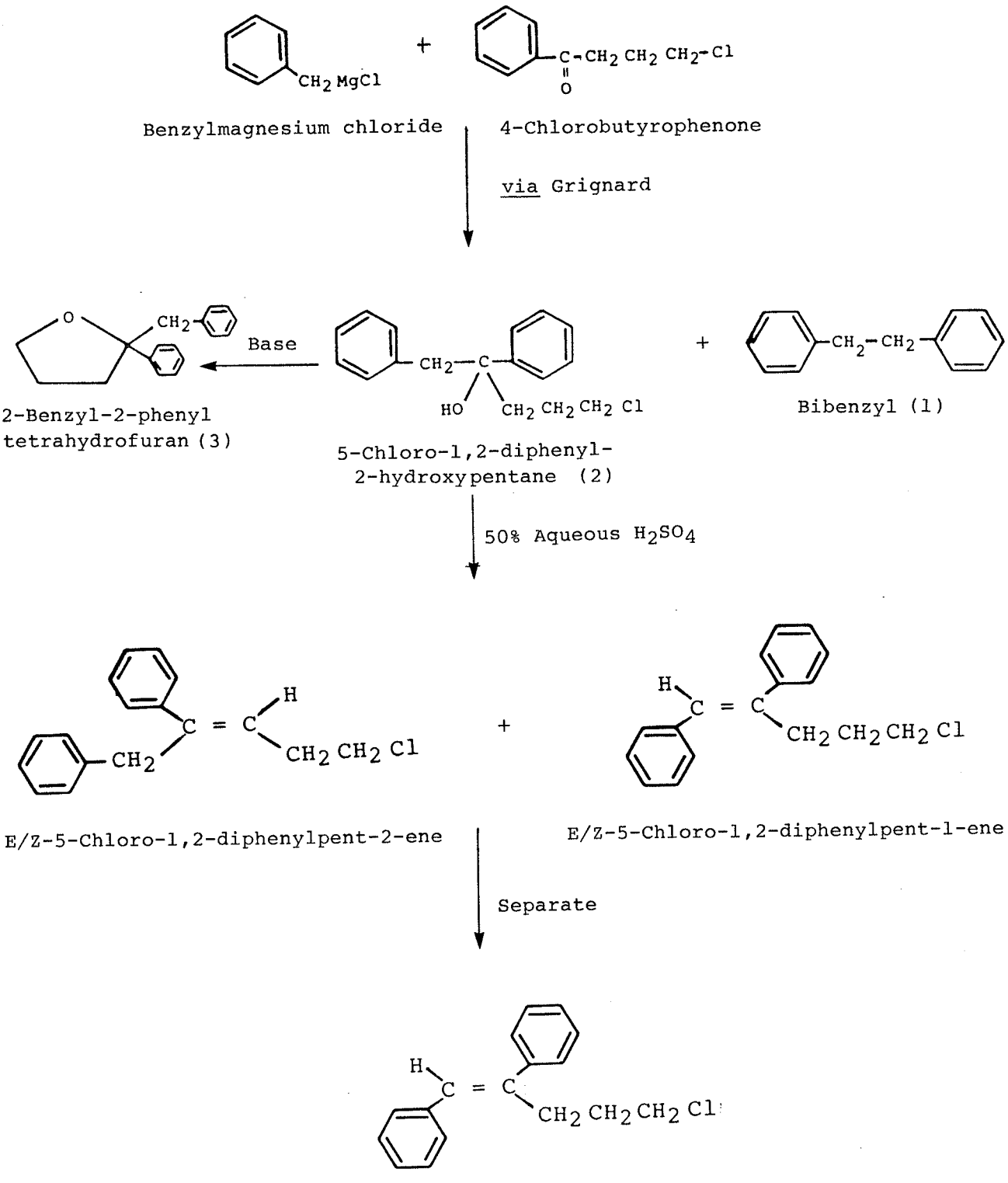
IV. SYNTHESIS

The objective of this project was to synthesize compounds with the potential to interact with the drug binding site located between residues 82-93 near calcium binding domain III on calmodulin (Fig. 11). The compounds would be structural variants of known calmodulin antagonists possessing unsubstituted hydrophobic groups separated by an alkyl chain from a basic substitutent as shown in Fig. 8.

The synthetic scheme shown in Fig. 15 produced the four target compounds of interest. In this scheme, a Grignard reaction using benzylmagnesium chloride and 4-chlorobutyrophenone produced the tertiary alcohol 5-chloro-1,2-diphenyl-2-hydroxypentane as indicated by the loss of the carbonyl group and the presence of a hydroxyl group in the IR spectrum of the crude reaction product. Attempts to isolate 5-chloro-1,2-diphenyl-2-hydroxypentane on an alumina column gave two products on elution with a benzene/cyclohexane gradient. The first product to be eluted from the column was identified as bibenzyl.[59] The second product to be eluted from the column was identified as the ether 2-benzyl-2-phenyltetrahydrofuran, rather than the desired 5-chloro-1,2-diphenyl-2-hydroxypentane. It was suspected that the basic nature of the alumina column had cyclized the tertiary alcohol to the ether as shown in Fig. 16.

The presence of the cyclic ether was deduced from the ^{1}H NMR (Appendix, Figs. 3,4) which indicated that the benzy-

Synthetic Scheme



E-5-Chloro-1,2-diphenylpent-1-ene (4a)
Z-5-Chloro-1,2-diphenylpent-1-ene (4b)

Fig. 15 Continued

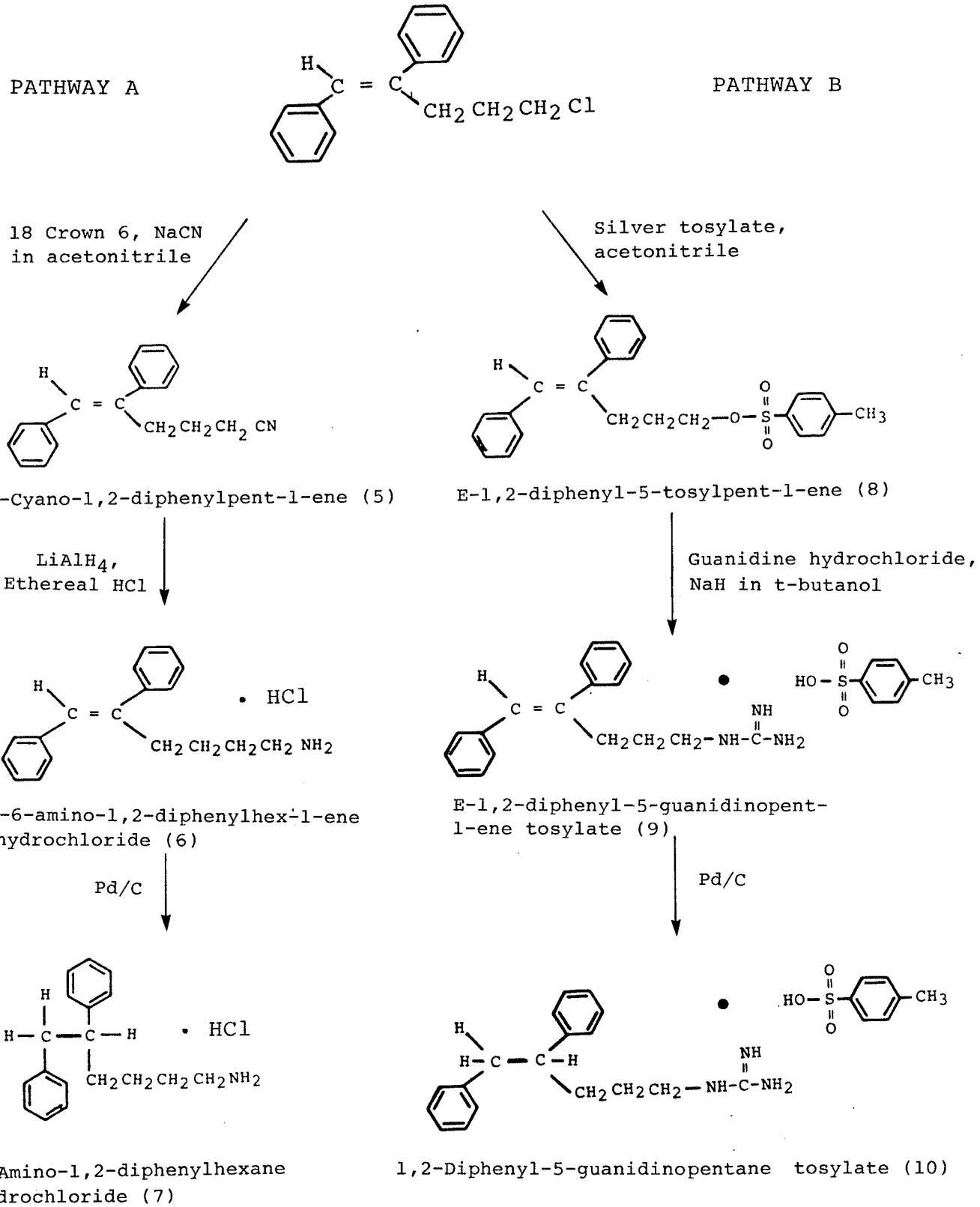
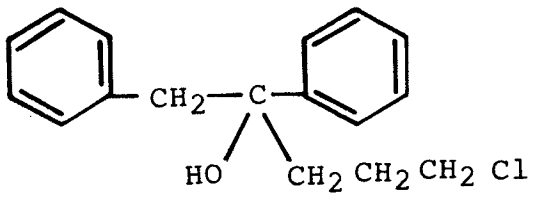
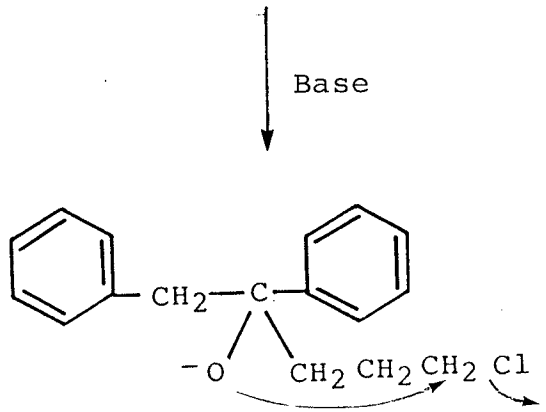
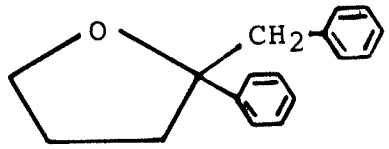


Fig. 15 Scheme for synthesis of potential calmodulin antagonists.



5-Chloro-1,2-diphenyl-2-hydroxypentane





2-Benzyl-2-phenyltetrahydrofuran

Fig. 16 Base catalyzed cyclization of 5-chloro-1,2-diphenyl-2-hydroxypentane to 2-benzyl-2-phenyl-tetrahydrofuran.

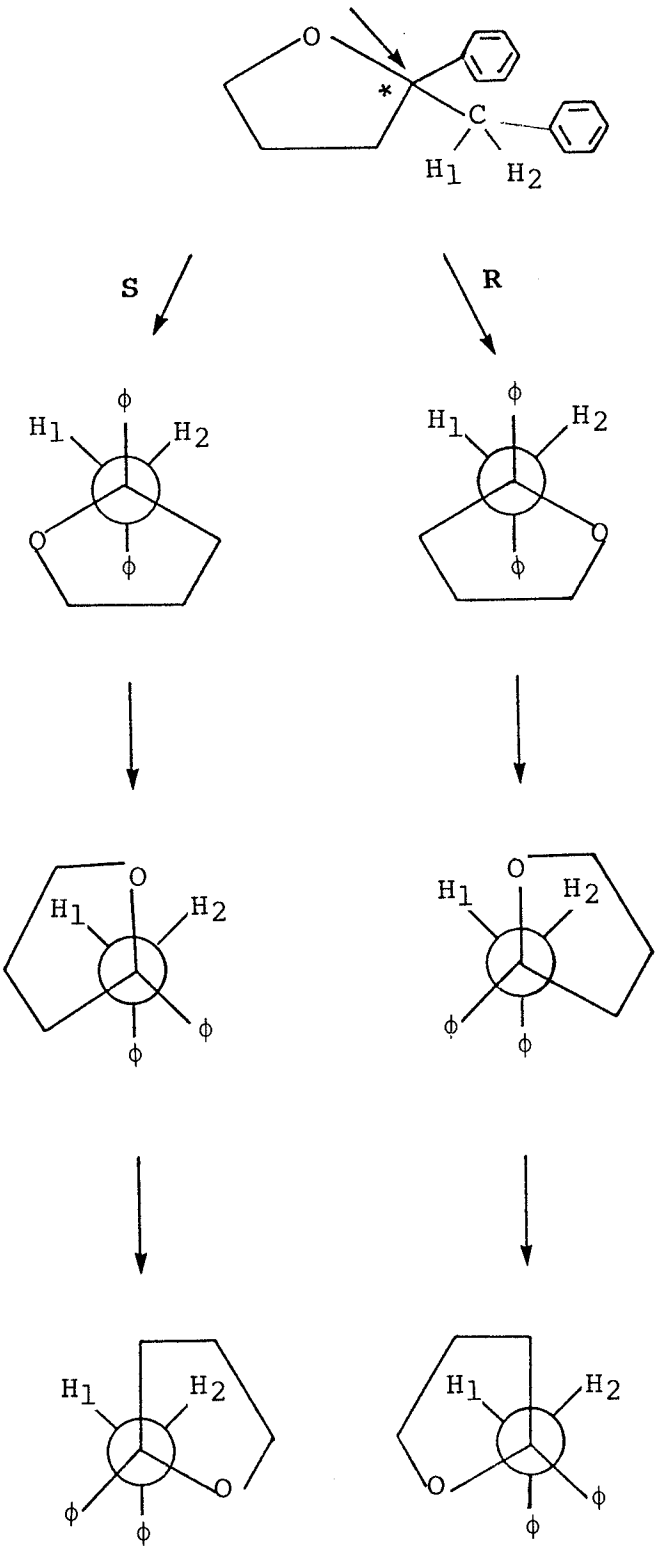


Fig. 17 Conformations of R and S 2-benzyl-2-phenyltetrahydrofuran.

lic protons were adjacent to a chiral center (denoted by an asterisk in Fig. 17). Examination of the Newman projection diagram in Fig. 17 of the R and S configurations[60] and their three possible staggered conformations shows that there are no mirror image environments for the benzylic protons. The benzylic protons H_1 and H_2 are not magnetically equivalent because C-2 is asymmetric. This results in the four peaks corresponding to the two protons at δ 3.05 ppm. Two peaks are due to each of the protons and two more due to geminal coupling.[61]

To avoid the problem of base catalyzed cyclization, the crude product mix containing the 5-chloro-1,2-diphenyl-2-hydroxypentane was dehydrated with 50% aqueous sulfuric acid[60] or p-toluenesulfonic acid monohydrate in benzene.[62] This elimination reaction produced a mixture of geometric isomers (Fig. 15) as detected by HPLC on the Novapak column (Figs. 18,19,20).

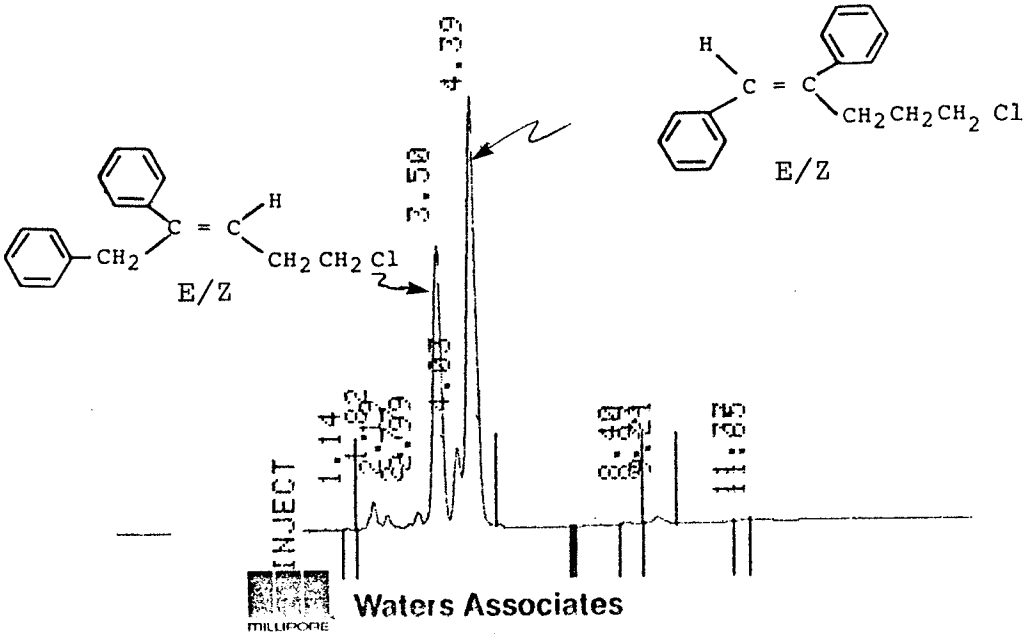


Fig. 18 HPLC elution profile (Novapak column) of the product mixture obtained from the acid catalyzed dehydration of 5-chloro-1,2-diphenyl-2-hydroxypentane. The two major peaks belong to E/Z-5-chloro-1,2-diphenyl-pent-2-ene (t = 3.5 minutes) and E/Z-5-chloro-1,2-diphenylpent-1-ene (t = 4.39 minutes).

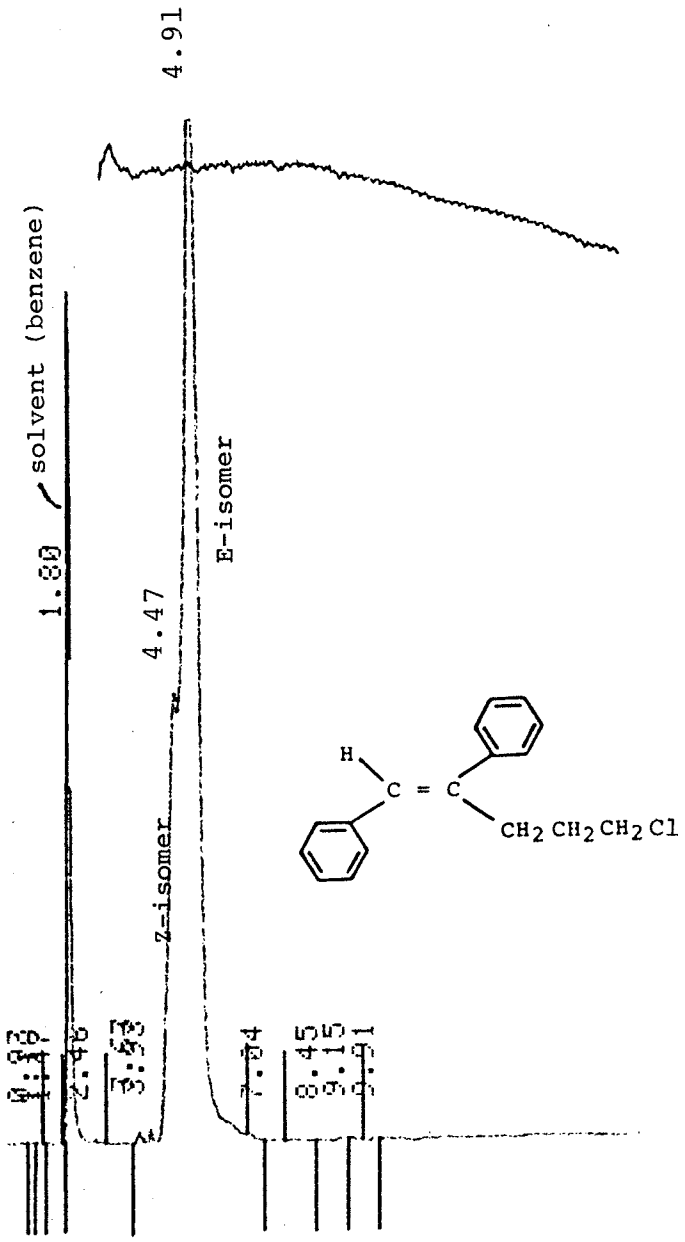


Fig. 19 HPLC elution profile (Novapak column) of E and Z 5-chloro-1,2-diphenylpent-1-ene from the crude reaction mixture. The reaction mixture was purified on a silica column using a benzene/cyclohexane gradient.

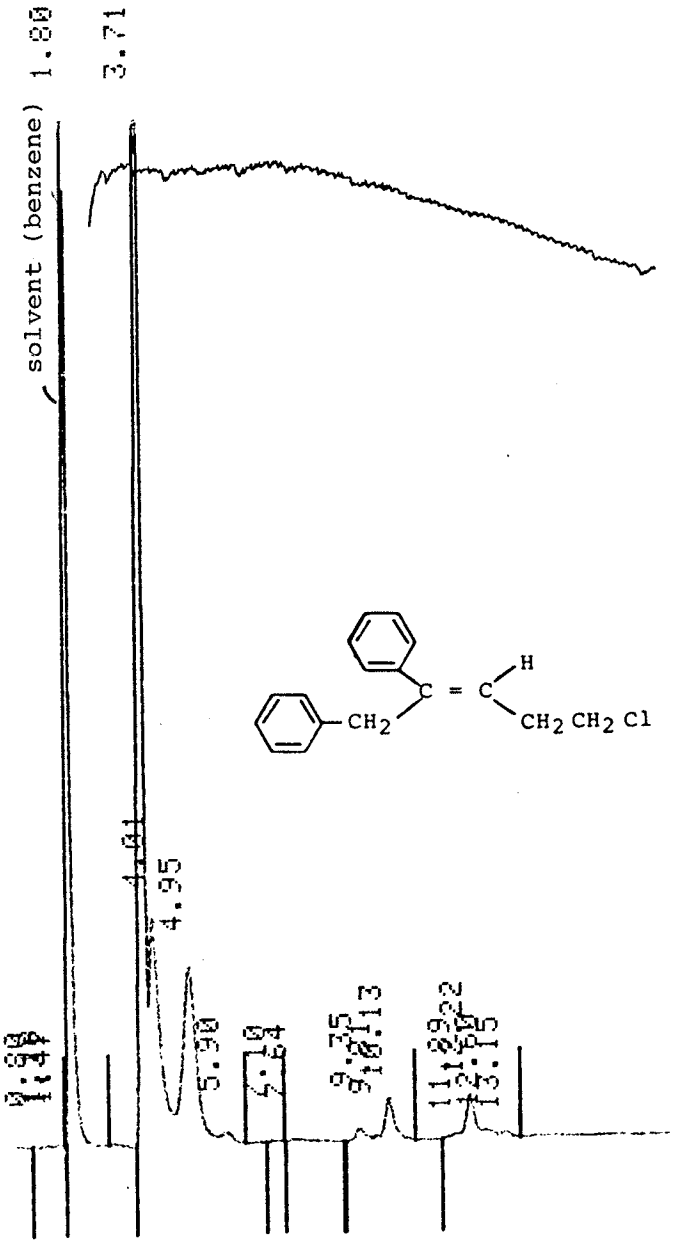


Fig. 20 HPLC elution profile (Novapak column) of E and Z 5-chloro-1,2-diphenylpent-2-ene from the crude reaction mixture. The reaction mixture was purified on a silica column using a benzene/cyclohexane gradient.

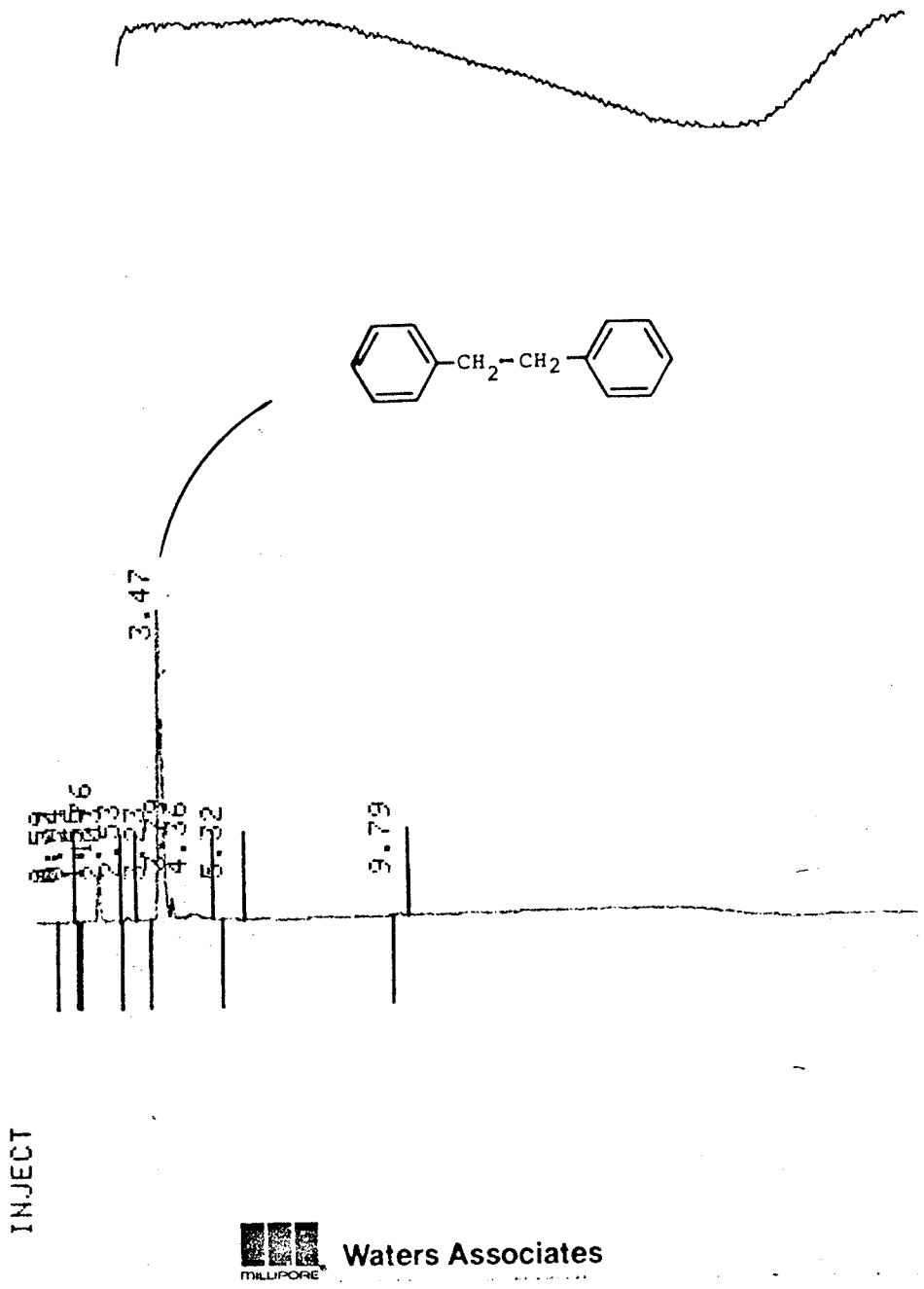
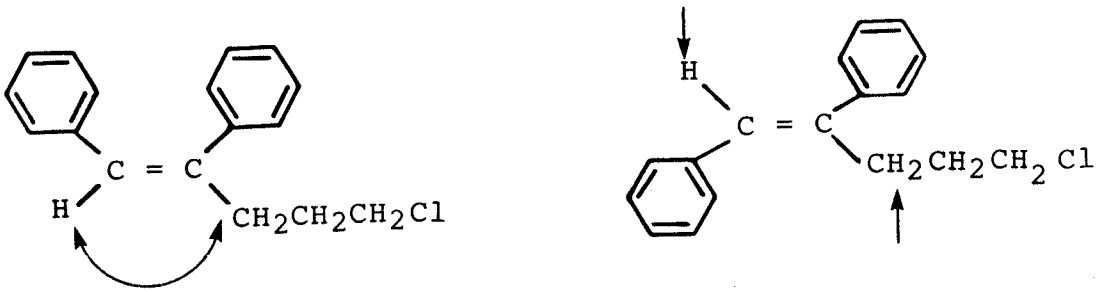


Fig. 21 HPLC elution profile of the by-product bibenzyl from the crude reaction mixture. The reaction mixture was purified on a silica column using a benzene/cyclohexane gradient.

The reaction mixture was purified on a silica column and the elution profile was monitored by HPLC which indicated the presence of three compounds which were collected, analyzed by NMR and identified as bibenzyl (t=3.40 minutes, Fig. 21, Appendix, Fig. 2), E/Z-5-chloro-1,2-diphenyl-pent-1-ene (t=4.39 minutes, Fig. 19, Appendix, Figs. 8-14) and E/Z-5-chloro-1,2-diphenyl-pent-2-ene (3.50 minutes, Fig. 20, Appendix, Fig. 25).

The HPLC profile of the crude product mixture (Fig. 18) indicated a peak height ratio of 1.6:1 in favour of the E/Z-5-chloro-1,2-diphenylpent-1-ene isomer. The fraction containing E/Z-5-chloro-1,2-diphenylpent-1-ene was analyzed on the Bruker (AM 300 mHz) NMR which detected two singlets at δ 6.50 and 6.78 ppm. These resonances were attributed to the vinyl protons in the Z and E isomers respectively (Appendix, Figs. 7, 8, and 12). E-5-chloro-1,2-diphenylpent-1-ene was expected to be the major product because the conjugated phenyl rings were <u>trans</u> with the carbon-carbon double bond giving a resonance stabilized structure which was also sterically less hindered and therefore more stable.[63] From the integration, the ratio of the Z to E isomer was found to be 1 : 4 (Appendix, Fig. 7). This fraction was further purified on the Altex semi-preparative column, the E(t=20.55 minutes) and Z (t=19.57 minutes) were separated, collected and analyzed by NMR. The configuration assigned to each isomer was confirmed using the nuclear Overhauser effect (NOE) as shown (Appendix Figs. 8 through 14).

The nuclear Overhauser effect provides information on configurations and conformations[64] by relating the NMR absorption of protons within close spatial proximity. The NMR spectra of both E and Z-5-chloro-1,2-diphenylpent-1-ene were first recorded and examined to determine where the vinyl proton appeared in the spectrum of each isomer. The vinyl proton was irradiated with the radiofrequency energy at the frequency of the vinyl proton and the methylene protons observed to determine if there was a change in their signal intensity. The methylene protons in the E isomer showed no observable effect while the methylene protons in the Z isomer indicated signal enhancement. Signal enhancement is due to the closer proximity of the vinyl proton to the methylene protons in the Z isomer but not in the E isomer as shown in Fig. 22.[64] (Appendix, Figs. 7 through 14).



NOE observed

Z isomer

NOE not observed

E isomer

Fig. 22 E and Z isomers of 5-chloro-1,2-diphenylpent-1-ene.

The four target compounds of interest were synthesized from E/Z-5-chloro-1,2-diphenylpent-1-ene. In pathway A (Fig. 15), an aliquot of the 4 : 1 (E:Z) mixture of 5-chloro-1,2-diphenylpent-1-ene was treated with sodium cyanide and 18-crown-6 in acetonitrile.[65] By increasing the solubility of NaCN in the acetonitrile, the phase transfer catalyst (18-crown-6) increased the rate of the Sn2 reaction of CN^- with the alkyl chloride (solid to liquid phase transfer) to give E/Z-5-cyano-1,2-diphenylpent-1-ene. This mixture was purified by recrystallization from ether/ethanol to give a pure sample of the E-isomer as determined by NMR. The spectrum did not show any traces of a singlet at δ 6.5 ppm corresponding to the vinyl proton of the Z-isomer indicating that only E-5-cyano-1,2-diphenylpent-1-ene had been isolated in pure form by recrystallization (Appendix , Fig. 15). The nitrile function in E-5-cyano-1,2-diphenylpent-1-ene was selectively reduced with lithium aluminum hydride to give E-6-amino-1,2-diphenylhex-1-ene.[66]

The carbon-carbon double bond in E-6-amino-1,2-diphenylhex-1-ene hydrochloride was reduced by catalytic hydrogenation over Pd/C catalyst to give the second target compound 6-amino-1,2-diphenylhexane hydrochloride.[66]

In pathway B (Fig. 15), an aliquot of the 4 : 1 (E:Z) mixture of 5-chloro-1,2-diphenylpent-1-ene was treated with silver tosylate in acetonitrile.[67] The resulting product was purifed by recrystallization from ethanol/ether to give

a pure sample of E-1,2-diphenyl-5-tosylpent-1-ene as determined by NMR. The spectrum (Appendix , Figs. 18, 19) showed a multiplet shifted downfield at δ 7.7 ppm for the two protons adjacent to the $-SO_2$ group in the aromatic region (δ 7.3-7.7 ppm). The singlet at δ 6.7 ppm indicated the vinyl proton in the E-configuration. Multiplets at δ 4.0, 2.7 and 1.7 ppm corresponded to the three sets of methylene protons and a singlet at δ 2.4 was attributed to the $-CH_3$ on the tosyl function. There was no evidence of a vinyl proton at δ 6.5 ppm indicating that the Z-isomer was not present and that only E-1,2-diphenyl-5-tosylpent-1-ene had been isolated by recrystallization (Appendix , Fig. 18).

E-1,2-Diphenyl-5-tosylpent-1-ene was treated with guanidine in dry t-butanol under reflux to give the third target compound, E-1,2-diphenyl-5-guanidinopent-1-ene as the tosyl salt.[68] Reduction of the carbon-carbon double bond in E-1,2-diphenyl-5-guanidinopent-1-ene tosylate by catalytic hydrogenation gave the fourth and final target compound 1,2-diphenyl-5-guanidinopentane tosylate.

V. BIOLOGICAL ACTIVITY OF E-6-AMINO-1,2-DIPHENYLHEX-1-ENE ON SMOOTH MUSCLE LIGHT CHAIN KINASE (MLCK)

The precise mechanisms regulating smooth muscle contraction have not as yet been clearly defined. It is known that actin, myosin and tropomyosin are the major contractile proteins found in smooth muscle (Fig. 23).[69] It is also known that the Ca^{+2} ion is involved in initiating contraction in a variety of cells. The calcium binding protein calmodulin and the enzyme myosin light chain kinase (MLCK) have been shown to play a regulatory role in smooth muscle contraction.[2]

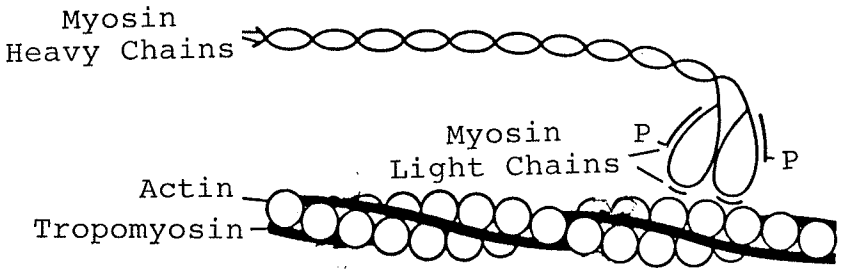


Fig. 23 Diagrammatic representation of the contractile proteins in smooth muscle.

Muscle contraction consists of the cyclic attachment and detachment of the globular head of the myosin molecule to the actin filament. When actin interacts with the myosin head it stimulates actin-myosin ATPase activity which causes the myosin and actin filaments to slide past each other produ-

cing a contraction.[69]

Actin is a globular protein (MW 43,000) which polymerises to form a double helical filament under physiological conditions. Tropomyosin is a fibrous protein (MW 66,000) composed of two alpha-helical subunits coiled about each other. Tropomyosin molecules lie end to end in the groove created by the two actin filaments (Fig. 23). Myosin, a very large molecule, is a hexamer and consists of two identical heavy chains (MW 200,000) and two pairs of lighter chains (MW 20,000 and 15,000) located near the globular head. The lighter chain of MW 20,000 can undergo reversible phosphorylation. The globular end of the myosin molecule contains the site of ATPase activity.[69]

The regulation of the actin-myosin interaction is shown in Fig. 24 and involves calcium, calmodulin and the highly specific Ca^{+2} dependent enzyme myosin light chain kinase.[69] The active kinase consists of two proteins.[70] One of these is a large protein of MW 134,000. It is responsible for the specificity of the enzyme and contains the catalytic site. The second protein is the calcium binding protein calmodulin (MW 16,000).

The sequence of events ultimately resulting in muscle contraction involves a stimulus causing a rise in the intracellular concentration of calcium. Calcium at a concentration of $10^{-5}M$ binds to calmodulin forming a Ca^{+2}-calmodulin complex. Since Ca^{+2} bound calmodulin has high affinity for

MLCK, it forms a complex in a 1:1 molar ratio with the enzyme. As a result of forming this complex, MLCK is changed to an active form which can then phosphorylate one of the light chains (MW 20,000) of myosin. This permits myosin to interact with actin to produce the contraction which persists as long as the concentration of Ca^{+2} is above the threshold level.[69]

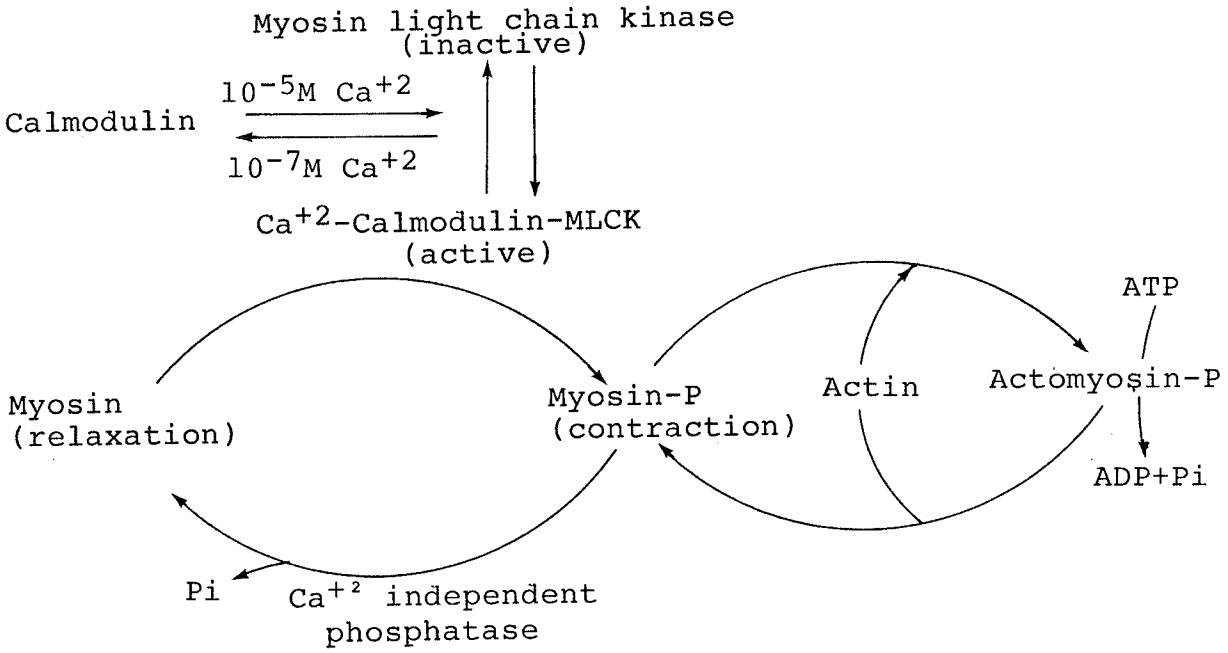


Fig. 24 Regulation of smooth muscle contraction by Ca^{+2} calmodulin and MLCK.[71]

The sequence of events is reversed with a drop in the level of intracellular Ca^{+2} from 10^{-5} to 10^{-7}M. The calcium-calmodulin complex dissociates from the active kinase resulting in an inactive form of MLCK which can no longer phosphorylate the lighter chain (MW 20,000) of myosin. A calcium independent phosphatase then dephosphorylates the light chain

of myosin and the muscle returns to its relaxed state.[69]

Recent studies have attempted to identify the calmodulin binding domain on MLCK.[72,73] and examine the validity of the regulatory mechanism between calmodulin and MLCK via Ca^{+2}.[74]

The study by Foyt et al.[72] treated purified chicken gizzard MLCK (130,000 Mr) with various proteases and analyzed the resulting fragments for their calmodulin binding properties. From their data a linear model for the functional calmodulin binding domain of MLCK was developed and indicated that it resided between the 85,000 to 87,000 kD region as shown in Fig. 25.

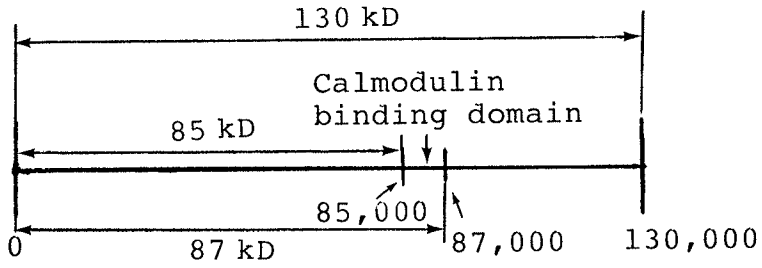


Fig. 25 Calmodulin binding domain on MLCK

A study in this area by Lukas et al.[73] resulted in isolation of a fragment, 20 amino acid residues in length from MLCK which possessed calmodulin binding properties. The amino acid sequence (NH_2-ARRKWQKTGHAVRAIGRLSS-COOH) of the peptide was determined and revealed a cluster of basic amino acid residues at the amino terminus. A peptide based on the native sequence (NH_2-RRKWQKTGHAVRAIGRLSS-COOH) was synthesized and

compared with the native fragment. Two dimensional projections of both peptides indicated that they contained a potential amphiphilic structure that had four arginines and one lysine on its polar face (Fig. 26). This was consistent with the recent suggestion that model peptides which bind to calmodulin with high affinity contain a potential amphiphilic helix structure.[75] It was further found that the synthetic peptide in fact adopted an α-helix structure upon binding to calmodulin and inhibited calmodulin activation of MLCK.

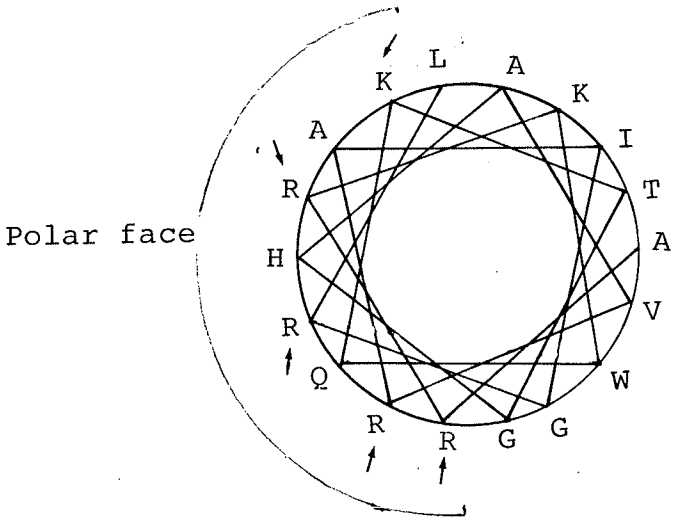


Fig. 26 Two dimensional structure of the peptide (NH_2-RRKWQKTGHAVRAIGRLSS-COOH) minus the carboxy terminal serines.[68]

An attempt was made to test the occurrence of this calmodulin binding structure in other proteins with known amino acid sequences. A 17 residue segment of the γ-subunit of skeletal muscle phosphorylase kinase, a calmodulin binding protein, was found to be homologous to the calmodulin binding

synthetic peptide based on the sequence found in MLCK. A synthetic peptide corresponding to the 17 residue segment (NH_2-REIVIRDFYACRPLRRL-COOH) of the γ-subunit did not possess significant calmodulin inhibitory activity. A more extended region of the γ-subunit (NH_2-RRVKPVTREIVIROPYALRPLRRL-COOH) possessing seven more amino acids at the amino terminus, of which three were basic, was synthesized and found to be a better inhibitor. These data suggested that calmodulin binding domains exhibit α-helix potential and contain extended basic amphiphilic regions. It is therefore possible that the interaction of calmodulin with its target enzymes may involve electrostatic interactions with charged basic residues in their calmodulin binding domains. The exact mode of the interaction between calmodulin and its binding site on MLCK, however, remains unknown as information on the precise primary amino acid sequence or three dimensional structure of MLCK is not as yet available.

A recent study[74] examined the validity of the regulatory role of MLCK on smooth muscle contraction via Ca^{+2}. As mentioned previously, calmodulin dependent MLCK is inactive at low Ca^{+2} levels. As Ca^{+2} concentration rises, it forms a Ca^{+2}-calmodulin complex possessing high affinity for MLCK. The complex binds to MLCK changing it to an active form which can exert its kinase activity. According to this mechanism, it is expected that once the Ca^{+2}-calmodulin complex has formed, it will remain active independently of Ca^{+2} concentration, that is, it will no longer be calcium sensitive.

It was proposed [74] that a fixed calmodulin-MLCK complex would be a valuable tool which could be used to test the calcium dependent mechanism of MLCK. A cross-linked calmodulin-MLCK complex was prepared and it was found that it was active independent of the calcium concentration. Further, it was found that chlorpromazine, a known calmodulin antagonist which interferes with the formation of the calmodulin-MLCK complex by competing for the binding site on calmodulin,[76] did not have any inhibitory effect on the crosslinked calmodulin-MLCK complex. From these experiments it was concluded that the calcium sensitivity of MLCK was probably responsible for regulating smooth muscle contraction. However, an *in vitro* system does not necessarily accurately reflect the situation *in vivo*. For example, there may be other calcium sensitive mechanisms present *in vivo* which may contribute to the activity of MLCK. Therefore, further studies are required in this area in a more organized system to further clarify the role of calcium in the regulation of smooth muscle contraction.

Thus far studies which attempt to locate the binding site for calmodulin on MLCK have been discussed. Conversely, attempts to localize this site of interaction for MLCK on calmodulin have shown that it lies between residues 78 and 148.[77] The study by Szyja *et al.* used affinity column chromatography to test binding of calmodulin fragments corresponding to residues 1-77(TR_1-C), 78-148 (TR_2-C) and 107-148 (TR_3-E)

to MLCK. The fragment TR_2-C was found to bind MLCK but failed to exert full activation when used in the same concentrations as calmodulin in an activity assay. The crystal structure and proteolysis studies of calmodulin indicate that the region extending between the second and third calcium binding loops on calmodulin (residues 68-92) is an α-helical conformation [37] which is exposed in the presence of calcium and hidden in the absence of calcium.[78] This α-helical region could possess the sites or part of the sites of interaction for the calcium dependent enzymes. Fragment TR_2-C possesses a part of this α-helical region which would explain its binding and partial activity with respect to MLCK.

As previously mentioned, many major tranquilizers are known to interact with calmodulin at two sites located near calcium binding domains I and III. The hypothetical drug binding site near calcium binding domain III extends from amino acid residues 82 to 92 and is a part of the amphiphilic α-helical region extending between calcium binding loops two and three thought to interact with calmodulin dependent enzymes.[77] A drug binding to this region could thus interfere with the interaction of calmodulin and its target enzyme.

The molecules, E-6-amino-1,2-diphenylhex-1-ene (compound 6), 6-amino-1,2-diphenylhexane (compound 7), E-1,2-diphenyl-5-guanidinopent-1-ene (compound 9) and 1,2-diphenyl-5-guanidinopentane (compound 10) shown in Fig. 15 were designed with two aromatic groups oriented to provide maximum interaction

with amino acid residues Phe 89 and Phe 92 in the hydrophobic region and a basic hydrophilic side chain to interact with Glu 87 in the acidic hydrophilic region of the putative drug binding site on calmodulin. The primary amine E-6-amino-1,2-diphenylhex-1-ene was assayed for its ability to inhibit phosphorylation of the light chain of myosin by calmodulin regulated myosin light chain kinase (MLCK).

In a preliminary study, the inhibitory activity of E-6-amino-1,2-diphenylhex-1-ene was compared with two reference compounds, tamoxifen and hydroxyzine known to inhibit calmodulin in this system (Fig. 27).[79] The IC_{50} of E-6-amino-1,2-diphenylhex-1-ene was found to be 50 μM. This was similar to the IC_{50} of 42 μM obtained from the inhibition of calmodulin regulated phosphodiesterase by chlorpromazine.[51]

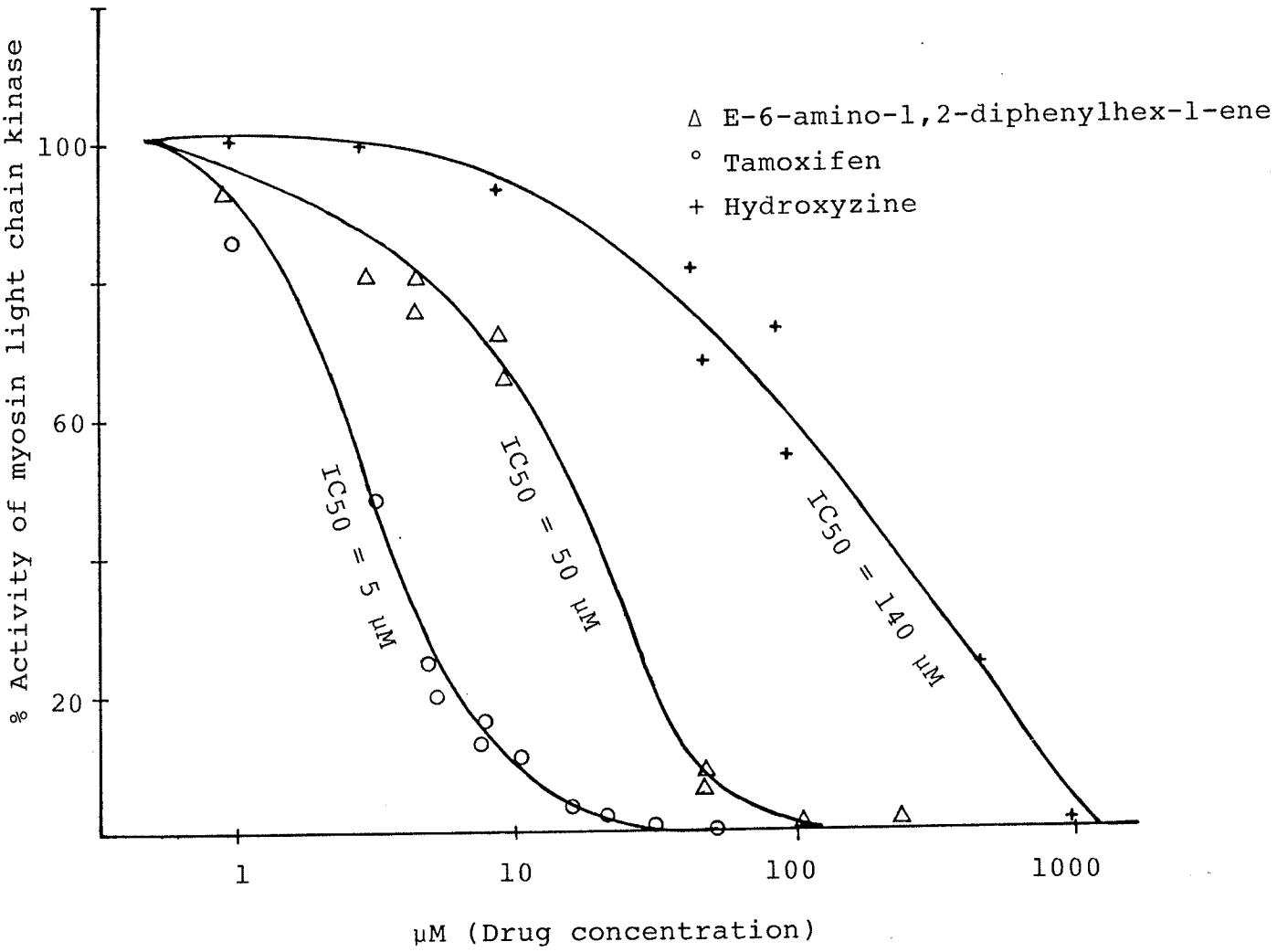


Fig. 27 Results indicating inhibitory activity of E-6-amino-1,2-diphenylhex-1-ene on myosin light chain kinase.

VI. CONCLUSION

This project describes the design and synthesis of potential calmodulin antagonists based on the hypothetical drug binding site located near calcium binding domain III (residues 80-113). One of the four compounds prepared, E-6-amino-1,2-diphenylhex-1-ene hydrochloride, was tested and shown to inhibit calmodulin regulated smooth muscle light chain kinase (MLCK) with an IC_{50} resembling that of chlorpromazine inhibition of calmodulin regulated phosphodiesterase. This preliminary study therefore lends support to the hypothesis that the drug binds in the manner proposed by the amphiphilic α-helix hypothesis as shown in Fig. 11.[38,39]

The compounds prepared were unsubstituted on the aromatic rings whereas the major tranquilizers shown in Fig. 6 are fluoro substituted. Pimozide, the most potent calmodulin inhibitor is fluoro substituted at the <u>para</u> position on both the aromatic rings (Fig. 6). It would therefore be interesting to synthesize structural analogues of the compounds prepared for this thesis which were fluoro or chloro substituted at the <u>para</u> position and observe their effect on calmodulin inhibition. Given that ring substitutions which increase the hydrophobicity of the aromatic region lead to more potent analogues[50,51], (CF_3 > F > Cl > H > CH_3)[51], it would be thought that the CF_3, fluoro or chloro substituted compounds would be the more effective calmodulin inhibitors. Similarly substitutions at the <u>ortho</u> and <u>meta</u> positions could also be made and tested.

The length of the alkyl chain could be extended to five or six carbon atoms to see if in fact a chain length of three or four carbon atoms is the optimum "spacer" between the hydrophobic region and the basic group. Varying the nature of the basic group by increasing or decreasing basicity and bulkiness would allow evaluation of its interaction with Glu 87 in the hydrophilic region of the protein. It should be kept in mind that substituents which are more basic may also be very bulky giving rise to the problem of steric hindrance in the hydrophilic region. Therefore a balance between the two would lead to a more effective interaction in this region.

A great deal of interesting chemistry and molecular manipulations can be a major part of this project. Future work in this area will involve testing the remaining three compounds prepared as a part of this thesis in the calmodulin regulated MLCK system. Based on these results, compounds with alterations to the aromatic region, alkyl chain and basic side chain can then be designed to improve binding to calmodulin.

Synthetic peptide analogs of the drug binding region can be prepared and drug-peptide interactions studied to try and clarify the nature of the interaction between drugs and calmodulin. Modification of the native peptide at residues proposed to bind the drug could ultimately delineate the amino acids involved in the interaction and may help in

understanding the role calmodulin plays in a variety of biochemical processes.

VII. EXPERIMENTAL

A. Synthetic Studies

Instruments

Melting points (m.p.) were determined with a Kofler dry stage melting point apparatus and are uncorrected.

Infrared (IR) spectra were recorded with a Perkin Elmer Model 267 Infrared spectrophotometer in CCl_4 (8 mg/0.8 ml) solution unless otherwise stated. Designations used in characterising absorption strengths in the IR spectra are, strong (s), medium (m) and weak (w).

Proton magnetic resonance (^1H NMR) spectra were recorded using deuteriochloroform ($CDCl_3$) as the solvent and tetramethylsilane (TMS) as the internal standard unless otherwise stated using the Bruker AM (300 mHz) NMR. Spectra were recorded at the Department of Chemistry, University of Manitoba, Winnipeg, Manitoba. The following designations are used in characterizing ^1H NMR signals: singlet (s), doublet (d), triplet (t), quartet (q) and multiplet (m).

Mass spectra (MS) were recorded on a Finnigan Quadrupole Model 1015 mass spectrophotometer at 70 eV using the direct probe method at the Department of Chemistry, University of Manitoba.

Elemental analyses were performed by Mr. W. Baldeo at the Microanalytical Laboratory, School of Pharmacy, University of London England.

High pressure liquid chromatography (HPLC) was carried out using a Waters UGK Autoinjector, two Model 510 pumps, Model 730 data module, the Model 721 Programmable System Controller and Lambda Max Model 481 spectrophotometer. Absorbances were recorded at 250 nm, chartspeed 0.5 cm/min and absorbance units full scale (AUFS) at 1 unless otherwise stated. Columns used were: (a) Waters Novapak, reverse phase C-18, particle size 5 μm, 15 cm x 4.6 mm (i.d.) and (b) Altex Ultrasphere - ODS, reverse phase, particle size 5 μm, 25 cm x 10 mm (i.d.). The gradient used for the Waters Novapak column is given in Table 2.

Table 2

Gradient table for the Waters C-18 column

Time (min.)	Flow (ml/min)	%A (H_2O)	%B (CH_3OH)	Curve
0	1	15	85	
10	1	0	100	linear
12	1	0	100	linear
15	1	15	85	linear
30	1	15	85	linear

The gradient used for the Altex Ultrasphere - ODS column is given in Table 3.

Table 3

Gradient table for the Altex Ultrasphere-ODS column

Time (mins.)	Flow (ml/min)	%A (H_2O)	%B (CH_3OH)	Curve
0	2	10	90	-
10	2	10	90	linear
15	2	0	100	linear
21	2	0	100	linear
25	2	10	90	linear
40	2	10	90	linear

Retention times for the compounds purified on the HPLC are given in Table 4.

Table 4

Compounds purified on the HPLC

Compound	C-18	Altex
2-benzyl-2-phenyl-tetrahydrofuran (3)	2.88 min	14.60 min
5-Chloro-1,2-diphenyl-5-hydroxypentane (2)	3.69 min	15.10 min
E-5-Chloro-1,2-diphenyl-pent-1-ene (4a)	4.91 min	20.55 min
Z-5-Chloro-1,2-diphenyl-pent-1-ene (4b)	4.47 min	19.57 min

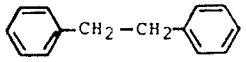
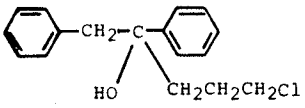
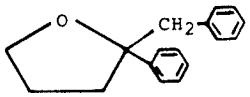
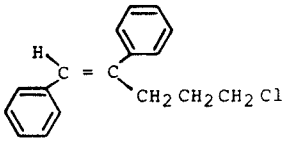
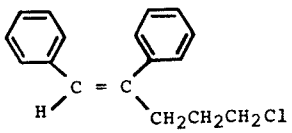
Materials

Thin layer chromatography was carried out on BDH precoated silica gel 60 F-254, 5 x 20 cm, 0.2 mm in thickness or Analtech precoated silica gel GHLF plates, 2.5 x 10 cm, 0.25 mm in thickness using one of the following developing solvents: (a) benzene or (b) CH_3Cl:CH_3OH:NH_4OH (75:25:2 drops). The R_f data are given in Table 5. The TLC plates were viewed under an ultraviolet source at 250 nm. An iodine chamber was subsequently used to visualize the products.

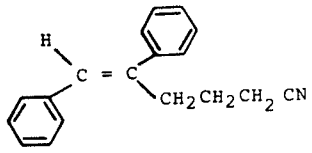
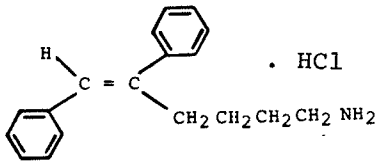
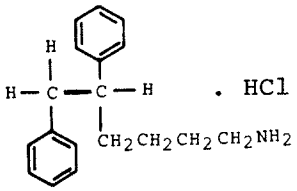
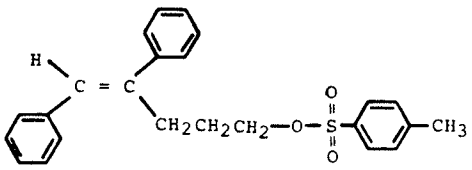
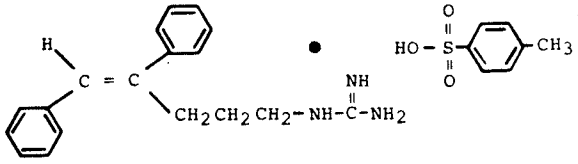
Column chromatography was carried out on an alumina (Brockman activity II aluminum oxide 90 for chromatographic adsorption analysis, BDH Chemicals) or Silica Gel 40 (70-230 mesh, Merck silica gel from BDH chemicals).

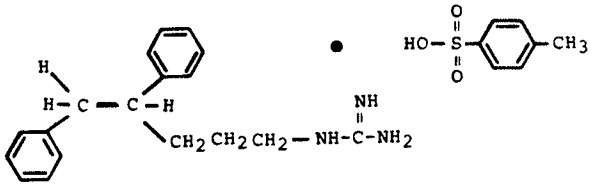
Table 5

R_f Data for compounds (1) to (10)

Compound	R_f BDH plate Developing system (a)	(b)	R_f Analtech plate Developing system (a)	(b)
 Bibenzyl (1)	0.59	—	0.86	—
 5-Chloro-1,2-diphenyl-2-hydroxypentane (2)	0.33	—	0.43	—
 2-Benzyl-2-phenyl-tetrahydrofuran (3)	0.31	—	0.57	—
 E-5-Chloro-1,2-diphenyl-pent-1-ene (4a)	0.60	—	0.86	—
 Z-5-Chloro-1,2-diphenyl-pent-1-ene (4b)	0.60	—	0.86	—

R_f Data for compounds (1) to (10)

Compound	R_f BDH plate Developing system (a)	(b)	R_f Analtech plate Developing system (a)	(b)
 E-5-Cyano-1,2-diphenyl-pent-1-ene (5)	—	—	0.49	—
 E-6-Amino-1,2-diphenylhex-1-ene hydrochloride (6)	—	—	—	0.53
 6-Amino-1,2-diphenyl-hexane hydrochloride (7)	—	—	—	0.58
 E-1,2-Diphenyl-5-tosyl-pent-1-ene (8)	—	—	0.44	0.95
 E-1,2-Diphenyl-5-guanidino-pent-1-ene tosylate (9)	—	—	—	0.29

Compound	R_f BDH plate Developing system (a)	(b)	R_f Analtech plate Developing system (a)	(b)
 1,2-Diphenyl-5-guanidino-pentane tosylate (10)	—	—	—	0.46

Bibenzyl (1)

Bibenzyl was formed as a by-product when the Grignard of benzyl chloride (3.0 g, 0.0237 mole) was treated with 4-chlorobutyrophenone (1.44 g, 0.0079 mole) to form 5-chloro-1,2-diphenyl-5-hydroxypentane (2). The reaction mixture was eluted from an alumina column (76 g, poured in cyclohexane) with cyclohexane to yield a solid (540 mg, 25% yield) which was recrystallized from ether/ethanol and gave m.p. 50.5-52°C (lit. m.p. 51-52°C[54]); MS m/z 182 (M^+), 91 ($M^+ - CH_2C_6H_5$), 77 (C_6H_5); NMR δ: 7.2(m), 2.92(s) ppm. (Appendix Fig. 2)

5-Chloro-1,2-diphenyl-2-hydroxypentane (2)

Benzyl chloride (41.58 g, 0.3285 mole was) added from a dropping funnel to a stirred mixture of magnesium turnings (7.98 g, 0.3285 mole) and a catalytic amount of iodine in anhydrous ether (300 mL) under argon in a triple necked 2 L flask fitted with a reflux condenser and overhead stirrer.

The reaction was started by adding benzyl chloride (10 mL) to the magnesium with gentle stirring. The solution was warmed and upon disappearance of the iodine color, heat was removed. The remaining benzyl chloride was added at a rate which kept the reaction mixture at reflux. When nearly all the magnesium had reacted (75 minutes), 4-chlorobutyrophenone (20 g, 0.1095 mole) was added slowly to the Grignard reagent *via* the dropping funnel and the mixture was maintained at reflux for 24 hours.

The reaction mixture was decomposed in the 2L flask with the dropwise addition of saturated NH_4Cl (250 mL) and the aqueous and organic layers separated. The aqueous layer was extracted with ether (3 x 100 mL).

The combined ether extracts and organic layer were washed with 5% sodium hydrogen sulfite (3 x 100 mL), 5% sodium hydrogen carbonate (3 x 100 mL), saturated NaCl (3 x 100 mL) and dried over anhydrous sodium sulfate.

The ether solution was filtered and evaporated to dryness at reduced pressure giving a pale yellow oil (63.97 g). An aliquot (500 mg) of the crude product containing 5-chloro-

1,2-diphenyl-5-hydroxypentane was purified on the HPLC using the Altex Ultrasphere-ODS semi-preparative column (Table 4) to give a clear oil, yield 150 mg, 30%; IR ν_{max}: 3580 cm^{-1} (O-H), (s); MS m/z: 274 (M^+), 197 ($M^+-C_6H_5$), 91 ($CH_2C_6H_5$), 77 (C_6H_5); NMR δ: 7.24 (m), 3.44(t), 3.16(q), 2.13(m), 1.86 (m) ppm (Appendix , Figs. 5,6).

Elemental analysis: $C_{17}H_{19}ClO$ requires %C 74.58, %H 6.63, %Cl 12.95: found %C 74.37, %H 6.71, %Cl 12.29.

2-Benzyl-2-phenyltetrahydrofuran (3)

Crude 5-chloro-1,2-diphenyl-2-hydroxypentane (1.92 g) was dissolved in cyclohexane (10 mL), loaded onto an alumina column (76 g, poured in cyclohexane) and eluted with a benzene /cyclohexane gradient.

100 mL cyclohexane
100 mL 1% benzene in cyclohexane
100 mL 2% benzene in cyclohexane
100 mL 3% benzene in cyclohexane
100 mL 4% benzene in cyclohexane
100 mL 5% benzene in cyclohexane
100 mL 10% benzene in cyclohexane

The elution was monitored by TLC (Table 5) and the fractions containing the product were collected, evaporated to dryness under reduced pressure yielding a colorless oil (750 mg, 40% yield). IR μ_{max}: 1050 cm^{-1} (C-O-C), (s); MS m/z: 147 ($M^+-CH_2C_6H_5$), 91 ($CH_2C_6H_5$), 77 (C_6H_5); NMR δ: 7.0-7.3(m), 3.9(m), 3.1(q), 2.1(m), 1.7(m) ppm (Appendix I, Figs. 3,4). Elemental analysis: $C_{17}H_{18}O$ requires %C 85.67, %H 7.61; found %C 85.87, %H 7.58.

2-Benzyl-2-phenyltetrahydrofuran was alternately prepared by treating crude 5-chloro-1,2-diphenyl-2-hydroxypentane (1.0 g, 0.0039 mole) with 1.2N potassium tertiary butoxide (50 mL) under reflux for 5.5 hours. The reaction was followed by TLC (Table 5) and was worked up by adding water (500 mL) followed by 1N HCl (75 mL). The solution was extracted with ether (4 x 100 mL). The ether extract was dried over anhydrous sodium sulfate, filtered and evaporated to dryness under reduced pressure to give an oil (700 mg, 93% yield)

which was purified on an alumina column (25 g, poured in cyclohexane) and then distilled to give 2-benzyl-2-phenyl-tetrahydrofuran (560 mg, 80% yield, NMR as above).

E- and Z-5-Chloro-1,2-Diphenylpent-1-ene (4a) and (4b)

Cooled (0°C) aqueous sulfuric acid (50% v/v, 150 mL) was added to crude 5-chloro-1,2-diphenyl-2-hydroxypentane (50.00 g), stirred (30 minutes), then refluxed (2 hours) and followed by TLC (Table 5). The reaction was poured into cold water (200 mL) and extracted with ether (3 x 100 mL). The combined ether extracts were washed with 5% sodium hydrogen carbonate (3 x 75 mL) and water (3 x 75 mL) then dried over anhydrous sodium sulfate. The ether extract was filtered and evaporated to dryness under reduced pressure and gave 44.43 g of crude product.

The crude mixture (8.00 g) was dissolved in cyclohexane (10 mL) and purified on a silica column (250 g, poured in cyclohexane) using a benzene/cyclohexane gradient as given below:

500 mL cyclohexane
500 mL 2% benzene in cyclohexane
4000 mL 5% benzene in cyclohexane

The separation was monitored by HPLC using the Waters Novapak C-18 column and gave bibenzyl (t=3.47 minutes, 1.68 g, 21% yield, white crystalline solid), then E/Z-5-chloro-1,2-diphenylpent-1-ene (t=4.91 minutes 4.00g, 50% yield, pale yellow oil) and E/Z-5-chloro-1,2-diphenylpent-2-ene (t=3.71 minutes, 2.4 g, 30% yield, pale yellow oil).

The fractions containing E/Z-5-chloro-1,2-diphenylpent-1-ene were collected and a 400 mg aliquot further purified on the HPLC using the Altex Ultrasphere-ODS semi-preparative column to give E-5-chloro-1,2-diphenylpent-1-ene (t=20.55

minutes, 200 mg, 50% yield, clear to pale yellow oil) and Z-5-chloro-1,2-diphenylpent-1-ene (t=19.57 minutes, 50 mg, 13% yield, clear to pale yellow oil). The fraction containing E-5-chloro-1,2-diphenylpent-1-ene was further distilled to give MS m/z: 256 (M^+), 207(M^+-CH_2Cl), 193 (M^+-CH_2CH_2Cl), 179 (M^+-$(CH_2)_3Cl$) 77 (C_6H_5); NMR δ: 7.26-7.48(m), 6.77(s), 3.49(m), 2.88(m), 1.90(m) ppm.(Appendix , Figs. 12, 13, 14).
Elemental analysis: $C_{17}H_{17}Cl$ requires %C 79.52, %H 6.68, %Cl 13.81; Found %C 79.79, %H 6.98, %Cl 13.74

Analysis of the Z isomer gave NMR δ: 6.94-7.30(m), 6.5(s), 3.56(m), 2.61(m), 1.86(m) ppm (Appendix , Figs. 8, 9, 10, 11).

E/Z-5-Chloro-1,2-diphenylpent-1-ene was also prepared by treating E/Z-5-chloro-1,2-diphenylpent-2-ene (1.5 g, 0.0059 mole) with p-toluene sulfonic acid monohydrate (1.0 g, 0.0053 mole) in benzene (100 mL) under reflux (60 minutes) to give a mixture of the geometric isomers E/Z-5-chloro-1,2-diphenylpent-1-ene and E/Z-5-chloro-1,2-diphenylpent-2-ene. The reaction was monitored by HPLC (Waters Novapak C-18 column) and after 60 mins. a 3:1 ratio in favour of the E/Z-5-chloro-1,2-diphenylpent-1-ene was obtained. Heat was removed, the reaction mixture cooled, 5% sodium hydrogen carbonate (200 mL) added and the organic and aqueous layers separated. The organic layer was washed with water (3 x 100 mL), dried over

anhydrous sodium sulfate, filtered and evaporated under reduced pressure to give a brown oil which was purified on a silica column to give E/Z-5-chloro-1,2-diphenylpent-1-ene (630 mg, 42% yield).

A second method for the dehydration of 5-chloro-1,2-diphenyl-2-hydroxypentane (2.0 g, 0.0073 mole) used p-toluenesulfonic acid monohydrate (1.0 g, 0.0053 mole in benzene (100 mL) under reflux (20 minutes) to give the mixture of geometric isomer E/Z-5-chloro-1,2-diphenylpent-1-ene and E/Z-5-chloro-1,2-diphenylpent-2-ene in a 1.2 to 1 ratio. The reaction was monitored by TLC (Waters Novapak C-18 column) and upon completion, heat was removed, the reaction mixture cooled, 5% sodium hydrogen carbonate (200 mL) added and the organic and aqueous layers separated. The organic layer was washed with water (3 x 100 mL), dried over anhydrous sodium sulfate and evaporated under reduced pressure to give E/Z-5-chloro-1,2-diphenylpent-1-ene and E/Z-5-chloro-1,2-diphenylpent-2-ene which could be separated on the silica column as before to give E/Z-5-chloro-1,2-diphenylpent-1-ene (800 mg, 40% yield) and E/Z-5-chloro-1,2-diphenylpent-2-ene (700 mg, 35% yield).

E-5-Cyano-1,2-diphenylpent-1-ene (5)

Sodium cyanide (4.6 g, 0.828 mole) and 18-crown-6 (396 mg) was added to E/Z-5-chloro-1,2-diphenylpent-1-ene (80:20, E/Z, 5.3 g 0.0207 mole) in acetonitrile (10 mL) and stirred at reflux for 36 hours. The reaction was monitored by TLC (Table 5), cooled, filtered and evaporated to one third its volume. Distilled water (10 mL) was added and the reaction mixture extracted with methylene chloride (3 x 30 mL). The methylene chloride extracts were combined, dried over anhydrous sodium sulfate, filtered and evaporated to dryness at reduced pressure to yield a brown solid residue (5.1 g, m.p. 62-63°C) which was recrystallized from ether/ethanol to give 3.2 g (63% yield, m.p. 66.5-68°C) of a white crystalline solid. IR ν_{max}: 2250 cm^{-1} (CN), (m); MS m/z 247 (M^+), 179 ($M^+-(CH_2)_3CN$), 77(C_6H_5); NMR δ: 7.25-7.46(m), 6.8 (s), 2.85(m), 2.25(m), 1.75(m) ppm (Appendix , Fig. 15). Elemental analysis: $C_{18}H_{17}N$ requires %C 87.41, %H 6.93, %N 5.66; found %C 87.33, %H 7.01, %N 5.92.

E-6-Amino-1,2-diphenylhex-1-ene hydrochloride (6)

E-6-Cyano-1,2-diphenylpent-1-ene (750 mg, 0.0030 mole) was added to a stirred solution of $LiAlH_4$ (807 mg, 0.0213 mole) in anhydrous ether (40 mL) in a 100 mL round bottom flask and maintained at reflux for 2 hours. The reaction was cooled in an ice bath and the excess $LiAlH_4$ destroyed by adding water (0.8 mL added dropwise), followed by 15% w/v NaOH (0.8 mL added dropwise) and water (2.4 mL added dropwise. The resulting precipitate was removed by filtration and the filtrate was washed with anhydrous ether (3 x 40 mL). The ether washes were collected, dried over anhydrous sodium sulfate, filtered and evaporated to dryness under reduced pressure to give a yellow oil. The yellow oil was redissolved in ether (25 mL) and acidified with ether saturated with HCl (15 mL) to produce the hydrochloride salt of E-6-amino-1,2-diphenylhex-1-ene (323 mg, 38% yield) which was recrystallized from ethanol/ether. m.p. 188-190°C; IR ν_{max} 1030 and 1070 cm^{-1} (C-N), (m), 3400 cm^{-1} (N-H), (w); MS m/z: 251 (M^+-HCl), 235 (M^+-NH_2.HCl), 179 (M^+-$(CH_2)_4NH_2$.HCl), 77 (C_6H_5); NMR (in D_2O) δ: 7.23-7.28(m), 6.72(s), 2.65(m), 1.38(m) ppm (Appendix , Fig. 16).

Elemental analysis: $C_{18}H_{22}Cl$ requires %C 75.11, %H 7.70, %N 4.86; found %C 74.92, %H 7.66, %N 4.68.

6-Amino-1,2-diphenylhexane hydrochloride (7)

Palladium on charcoal (10%, 50 mg) was added to E-6-amino-1,2-diphenylhex-1-ene hydrochloride (75 mg, 0.00026 mole) in absolute ethanol (50 mL) and stirred under hydrogen at atmospheric pressure for 16 hours. The product was filtered through Celite (prewashed with ethanol), evaporated to dryness under reduced pressure to yield a solid which was recrystallized from ethanol/ether. Yield 30 mg, 40%. m.p. 163-164°C; MS m/z: 253 (M^+-HCl); NMR δ: 7.00-7.23(m), 2.83(m), 1.58(m), 1.2(m) ppm (Appendix , Fig. 17).
Elemental analysis: $C_{18}H_{24}Cl$ requires %C 74.55, %H 8.34, %N 4.83; found %C 74.79, %H 8.40, %N 5.05.

E-1,2-Diphenyl-5-tosylpent-1-ene (8)

E/Z-5-Chloro-1,2-diphenylpent-1-ene (80:20, E:Z; 500 mg, 0.002 mole) in acetonitrile (5 mL) was added to silver tosylate[57] (1.330 g, 0.005 mole) in acetonitrile (5 mL) in a 25 mL round bottom flask fitted with a reflux condenser, stirred and maintained at reflux (protected from light with aluminum foil, 48 hours). The reaction was cooled to room temperature. Water (10 mL) was added, the mixture extracted with ether (3 x 20 mL) and the combined ether extracts dried over anhydrous sodium sulfate. The ether solution was filtered and evaporated to dryness under reduced pressure to leave a solid residue which was recrystallized from ether. Yield 374 mg, 48%; m.p. 90°C; IR ν_{max}: 1189 and 1190 cm^{-1} (R-O-SO_2-R), (s); MS m/z: 391 (M^+), 221 (M^+-tosyl), 207 (M^+-CH_2OTos), 193 (M^+-CH_2CH_2OTos), 179 (M^+- $(CH_2)_3OTos$), 77 (C_6H_5); NMR δ: 7.71(d), 7.26-7.38(m), 6.71(s), 3.98(t), 2.76(m), 2.44(s), 1.75(m) ppm (Appendix , Fig. 18, 19).

Elemental analysis: $C_{24}H_{24}SO_3$ requires %C 73.44, %H 6.16; found %C 73.38, %H 6.20.

E-1,2-Diphenyl-5-guanidinopent-1-ene tosylate (9)

Guanidine hydrochloride (162 mg, 0.0016 mole) was added to a stirred solution of sodium hydride (0.0016 mole, 64 mg of a 60% dispersion in oil) in anhydrous t-butanol (5 mL) and the mixture maintained at reflux for 30 minutes. The sodium chloride formed was filtered off and the filtrate added to a stirred solution of E-1,2-diphenyl-5-tosylpent-1-ene (300 mg, 0.008 mole) in t-butanol (5 mL) and maintained at reflux for 18 hours. The solvent was evaporated under reduced pressure to yield a solid which was extracted with boiling water. Upon cooling, the aqueous extract gave a white crystalline solid which was recrystallized form water. Yield 75 mg, 21%; m.p. 134-136°C; IR ν_{max}: 1685 and 1740 cm^{-1}(C=N), (m); MS m/z: 279 (M^+-TosOH), 221 (M^+-$CH_2NHC(=NH)NH_2$ and TosOH), && (C_6H_5); NMR δ: 7.61(d), 7.10-7.36(m), 6.72(s), 2.86(m), 2.75(m), 2.35(m), 1.65(m) ppm (Appendix , Figs. 20,21,22). Elemental analysis: $C_{25}H_{29}N_3O_3S$ requires %C 66.49, %H 6.47, %N 9.31; found %C 66.69, %H 6.59, %N 9.14.

1,2-Diphenyl-5-guanidinopentane tosylate (10)

Palladium on charcoal (10%, 50 mg) was added to 1,2-diphenyl-5-guanidinopent-1-ene tosylate (50 mg) dissolved in absolute ethanol (50 mL) and stirred under hydrogen at atmospheric pressure for 2 hours. The reaction mixture was filtered through Celite (prewashed with ethanol), evaporated to dryness under reduced pressure to yield a solid (30 mg, 60%), m.p. 105-106°C; MS m/z: 190 ($M^+-CH_2C_6H_5$); NMR δ: 7.6(m), 7.1-7.3(m), 2.8(m), 2.4(s), 1.4(m) ppm (Appendix A, Fig. 23). Elemental analysis: $C_{25}H_{31}N_3O_3S$ requires %C 66.20, %H 6.89, %S 7.07, %N 9.26; found %C 66.23, %H 6.80, %S 7.16, %N 9.32.

B. BIOLOGICAL STUDIES

Myosin Light Chain Kinase Assay

The assay[80] (Fig. 28) involved the isolation of myosin, actin, calmodulin and myosin light chain kinase from fowl gizzard smooth muscle.[81] Upon isolation, the calcium sensitive actomyosin complex was placed in a buffer mixture and the phosphorylation reaction started by the addition of radiolabelled ATP. The mixture was incubated for two minutes and at the end of this period, the reaction was stopped by the addition of cold 6% trichloroacetic acid containing 2% sodium pyrophosphate. The reaction mixture was washed, centrifuged and the resulting pellet suspended in a scintillation cocktail. The radioactivity recorded resulted from the radiolabelled phosphorylated light chain and was a measure of MLCK activity caused by calmodulin alone. This was the control experiment for the assay and determined full activity of the enzyme. The assay was repeated with the addition of varying concentrations of drug to determine the inhibition of light chain phosphorylation caused by the compound binding to calmodulin.

Turkey or chicken gizzard

↓ Extraction

Myosin,actin, calmodulin
myosin light chain kinase

↓

Calcium sensitive actomyosin complex

↓

1 mg actomyosin suspended in 1 ml buffer containing
5 mM Mg^{+2}
40 mM Imidazole buffer pH 7
50 mM KCl
10^{-5} Ca^{+2} in Ca-EGTA buffer

↓

Start reaction with the addition of radiolabelled ATP 1 μCi/ml. Incubate at 30°C for 2 minutes. MLCK phosphorylates the light chain (MW 20,000) with radiolabelled phosphate.

↓

Stop reaction with cold 6% trichloroacetic acid containing 2% sodium pyrophosphate. Wash reaction mixture with a. 6% TCA, b. acetone

↓ Centrifuge

Measure radioactivity of the pellet in scintillation counter to get activity of MLCK due to calmodulin regulation alone. This control experiment gives 100% activity of MLCK.

The assay is repeated by adding varying concentrations of drug to determine inhibition of light chain phosphorylation resulting from drug binding to calmodulin.

Fig. 28 Flow diagram of phosphorylation assay[80]

APPENDIX

^{1}H NMR of Compounds (1) to (10)

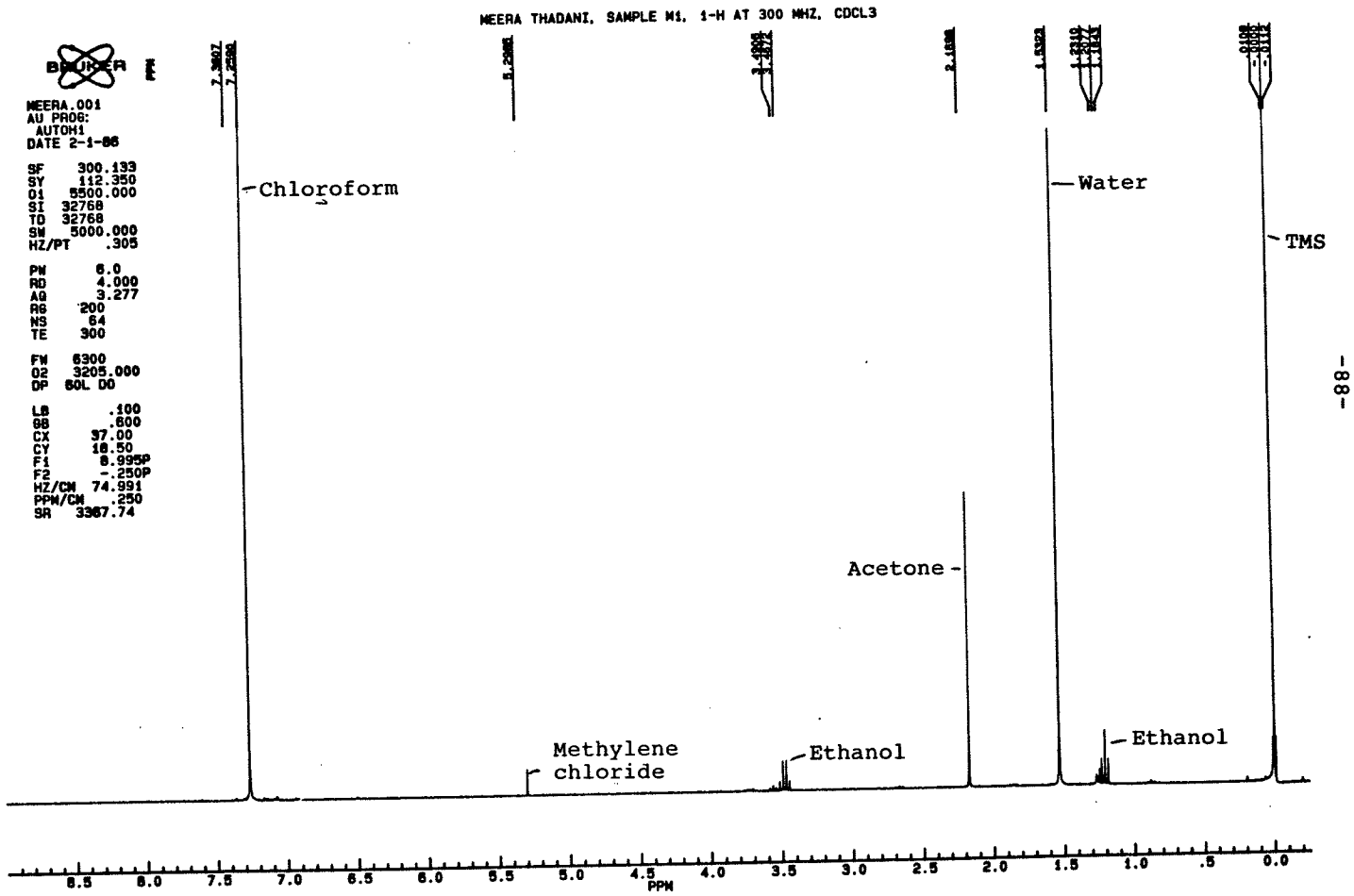


Fig. 1 Reference spectrum for solvent peaks.

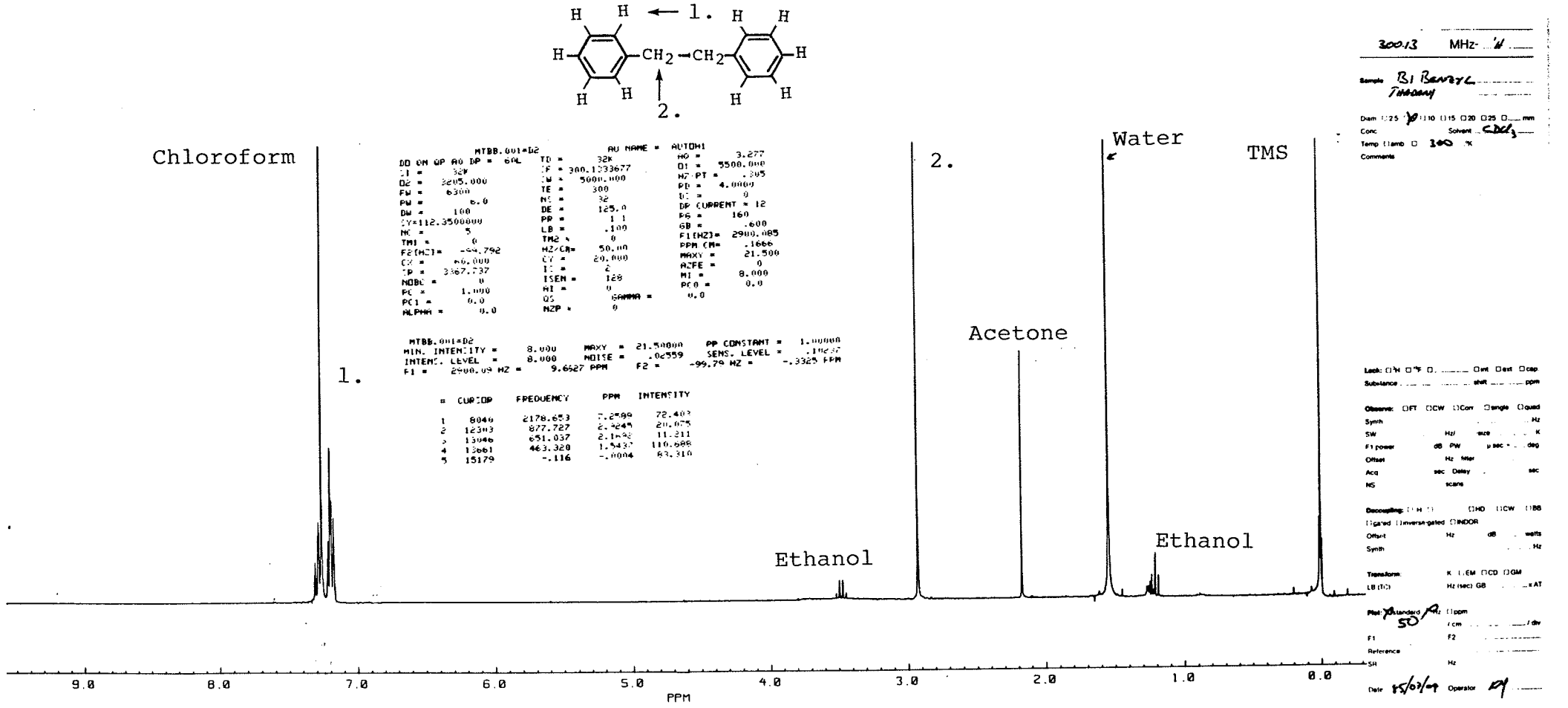


Fig. 2 Bibenzyl (1).

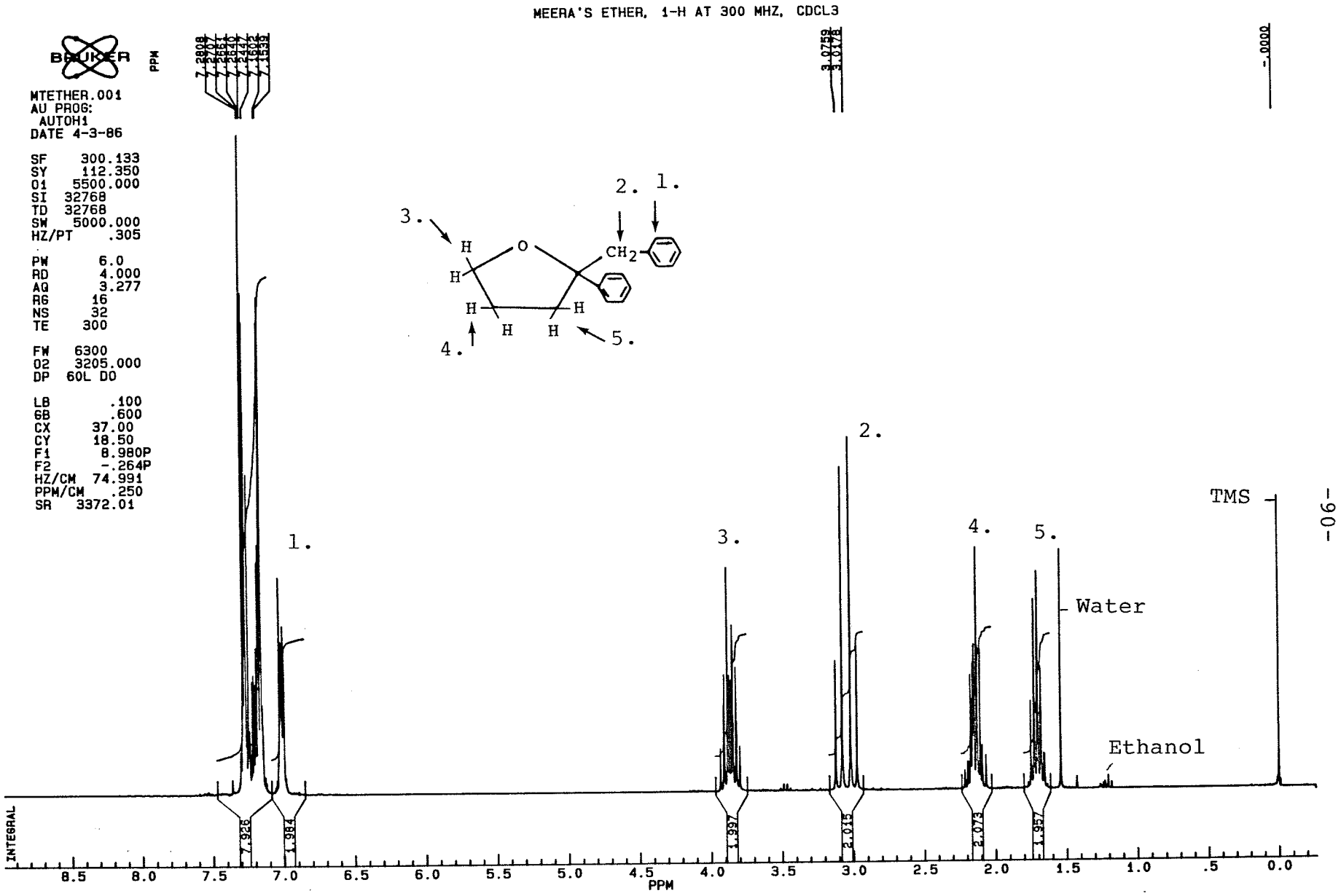


Fig. 3 2-Benzyl-2-phenyltetrahydrofuran (3)

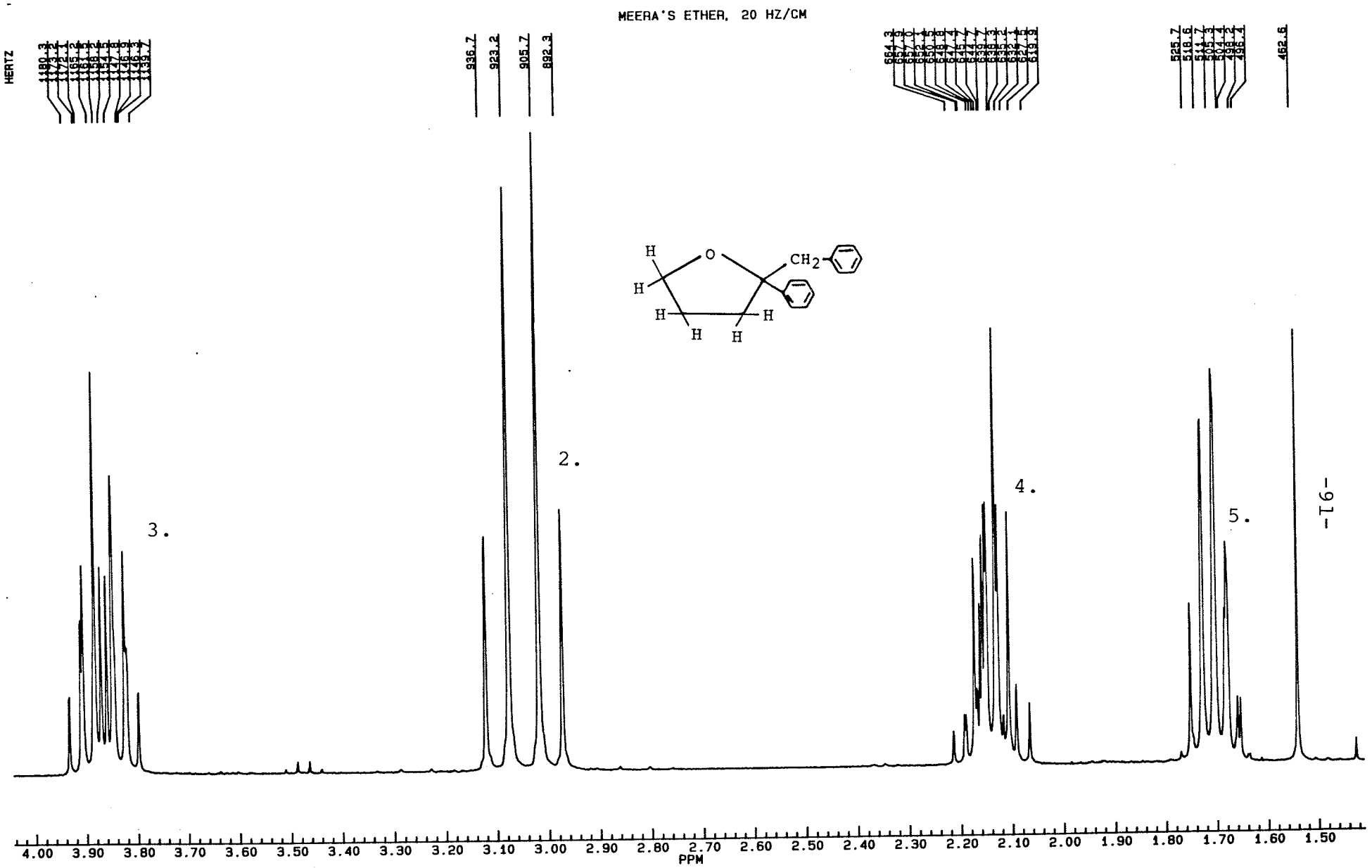


Fig. 4 2-Benzyl-2-phenyltetrahydrofuran (3)

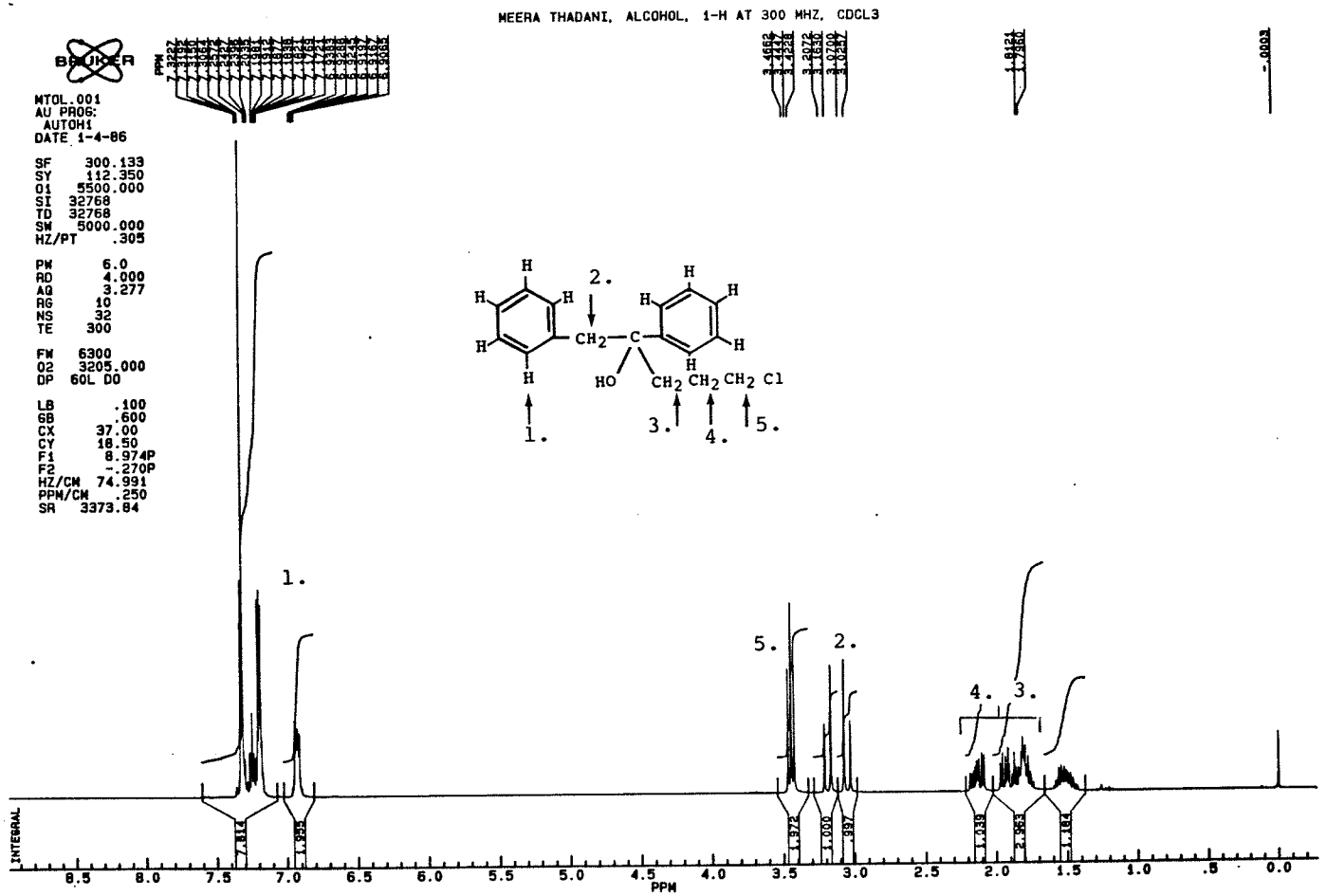


Fig. 5 5-Chloro-1,2-diphenyl-2-hydroxypentane (2)

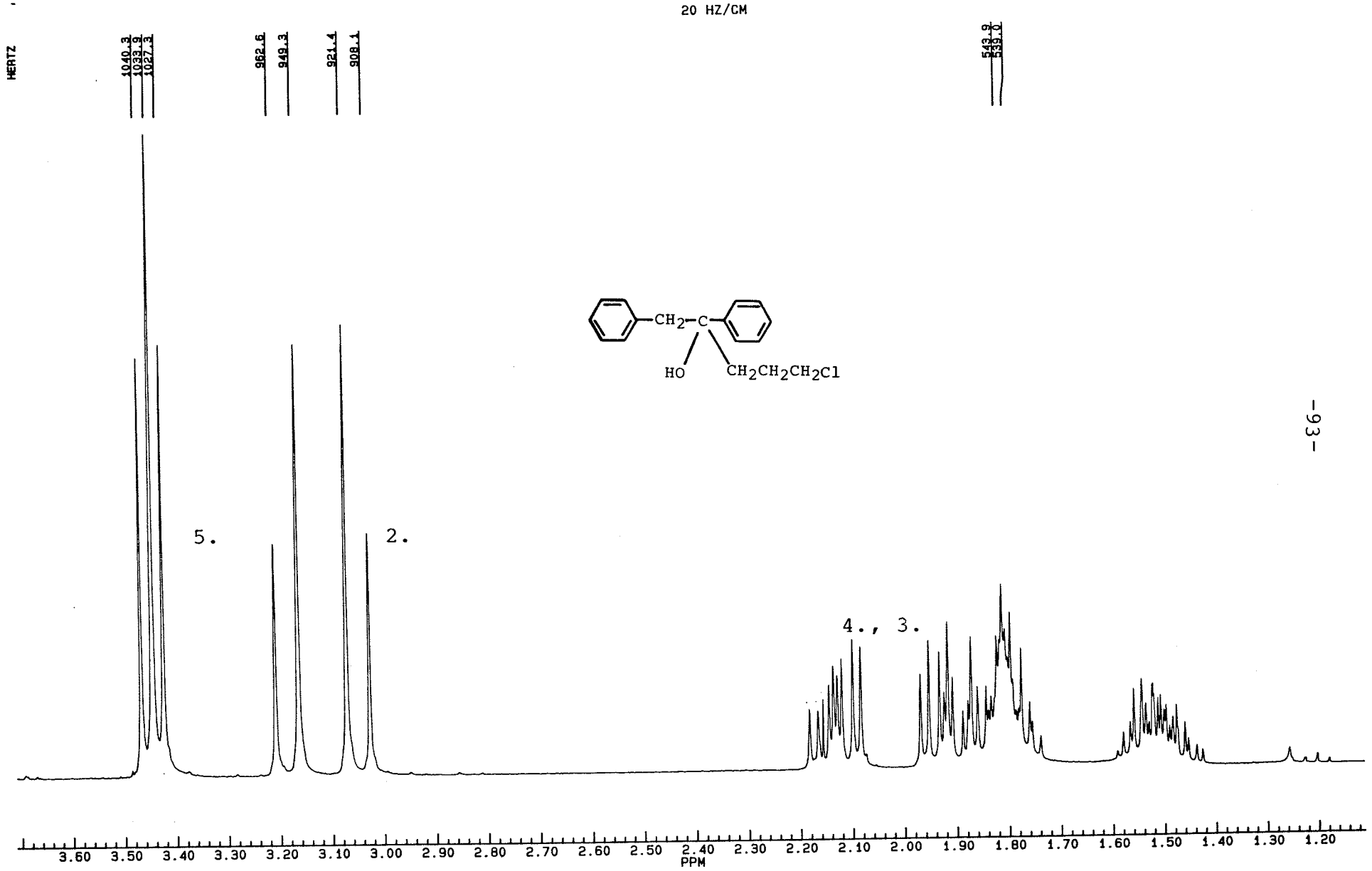


Fig. 6 5-Chloro-1,2-diphenyl-2-hydroxypentane (2)

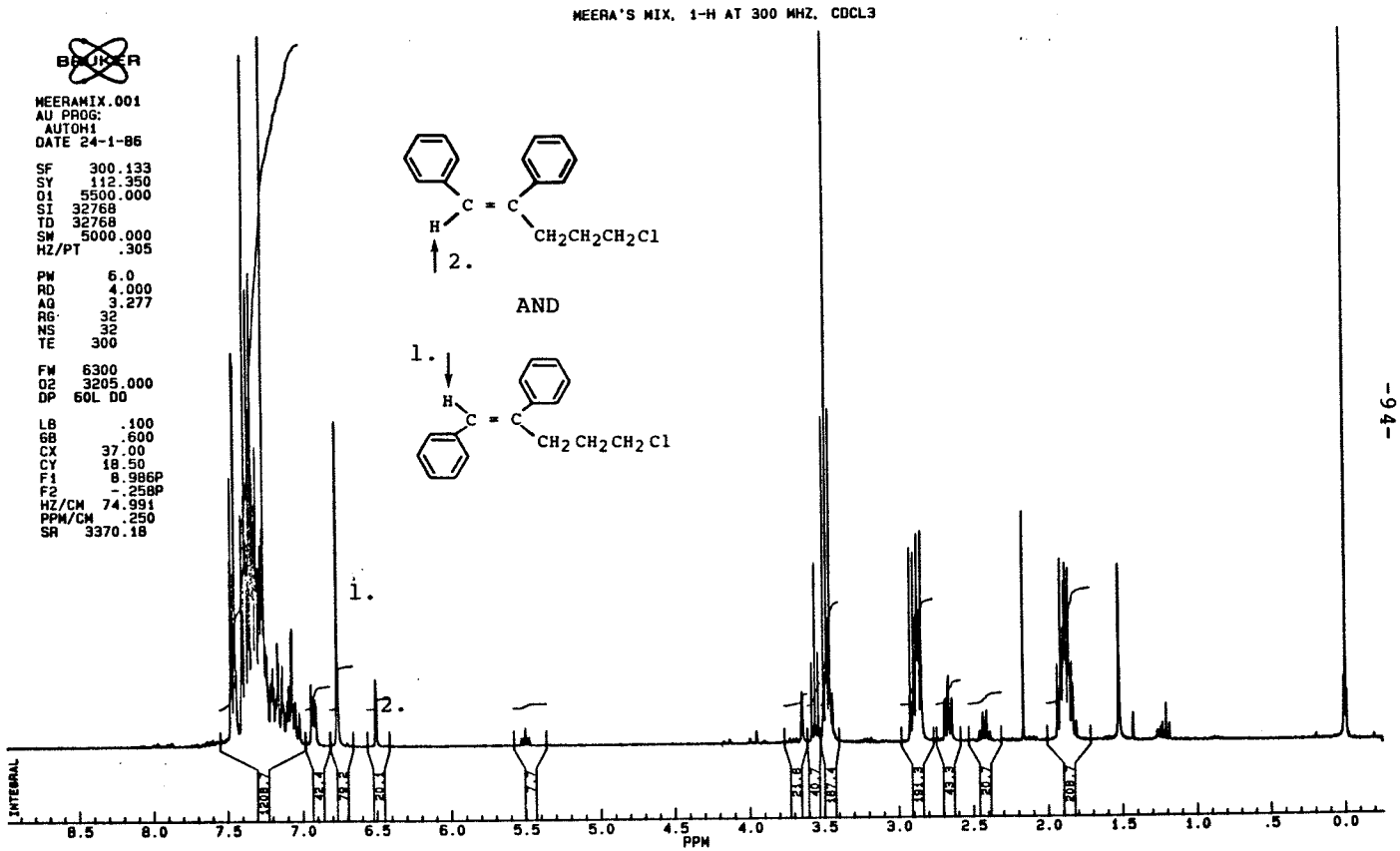


Fig. 7 E and Z 5-Chloro-1,2-diphenylpent-1-ene. The NMR indicates the presence of vinyl protons 1 and 2 and their respective ratios.

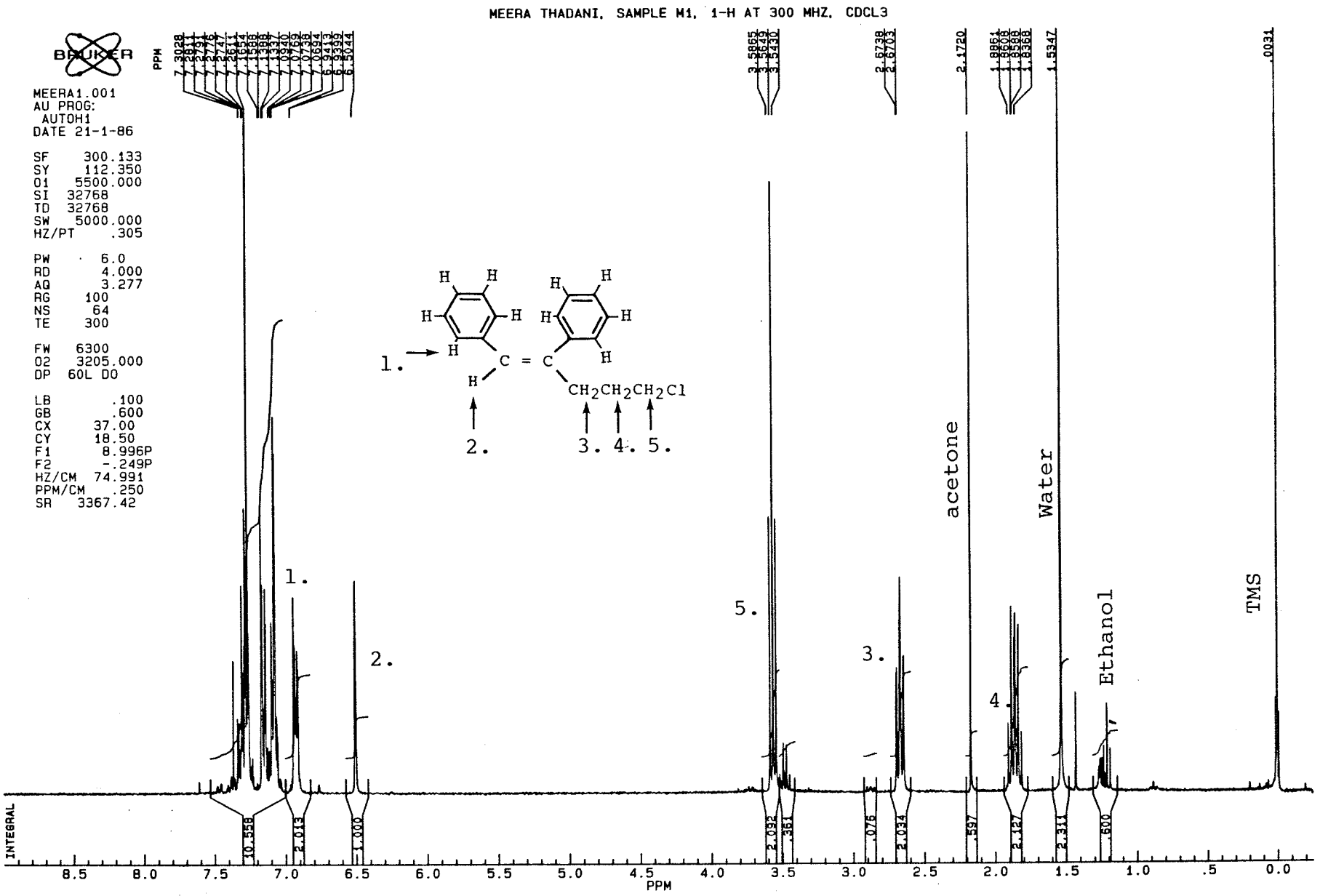


Fig. 8 Z-5-Chloro-1,2-diphenylpent-1-ene (4b)

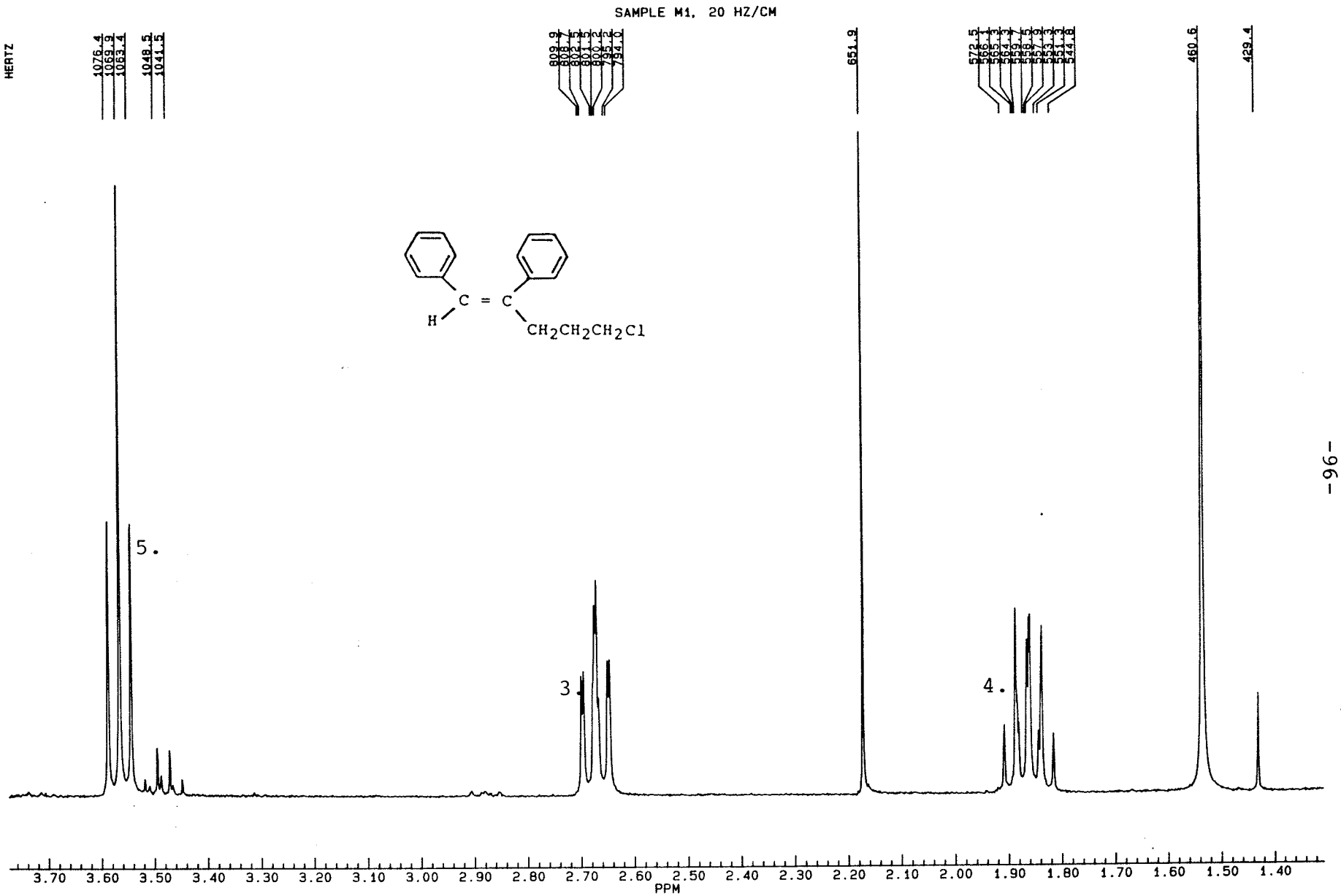


Fig. 9 Z-5-Chloro-1,2-diphenylpent-1-ene (4b).

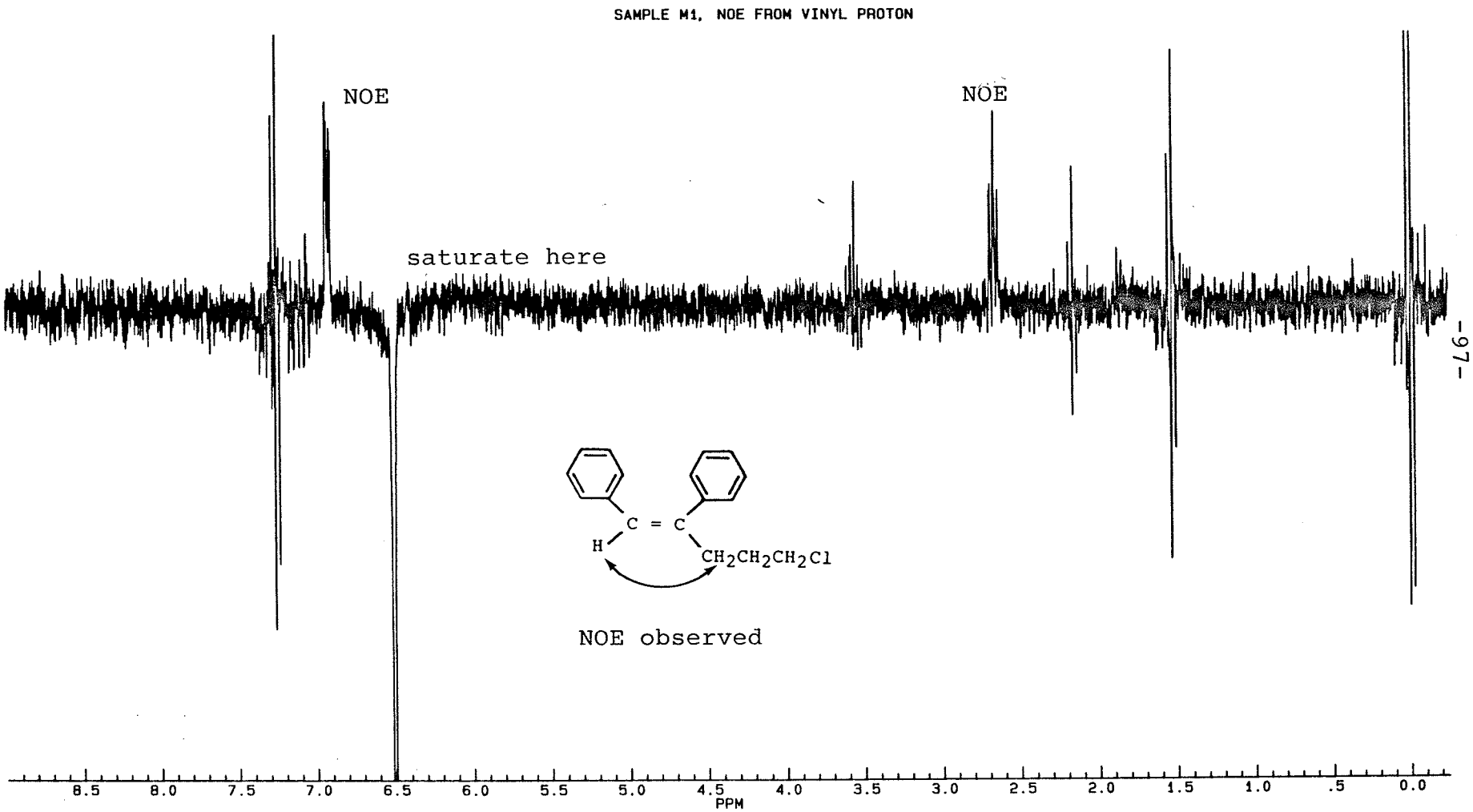


Fig. 10 Z-5-Chloro-1,2-diphenylpent-1-ene (4b).

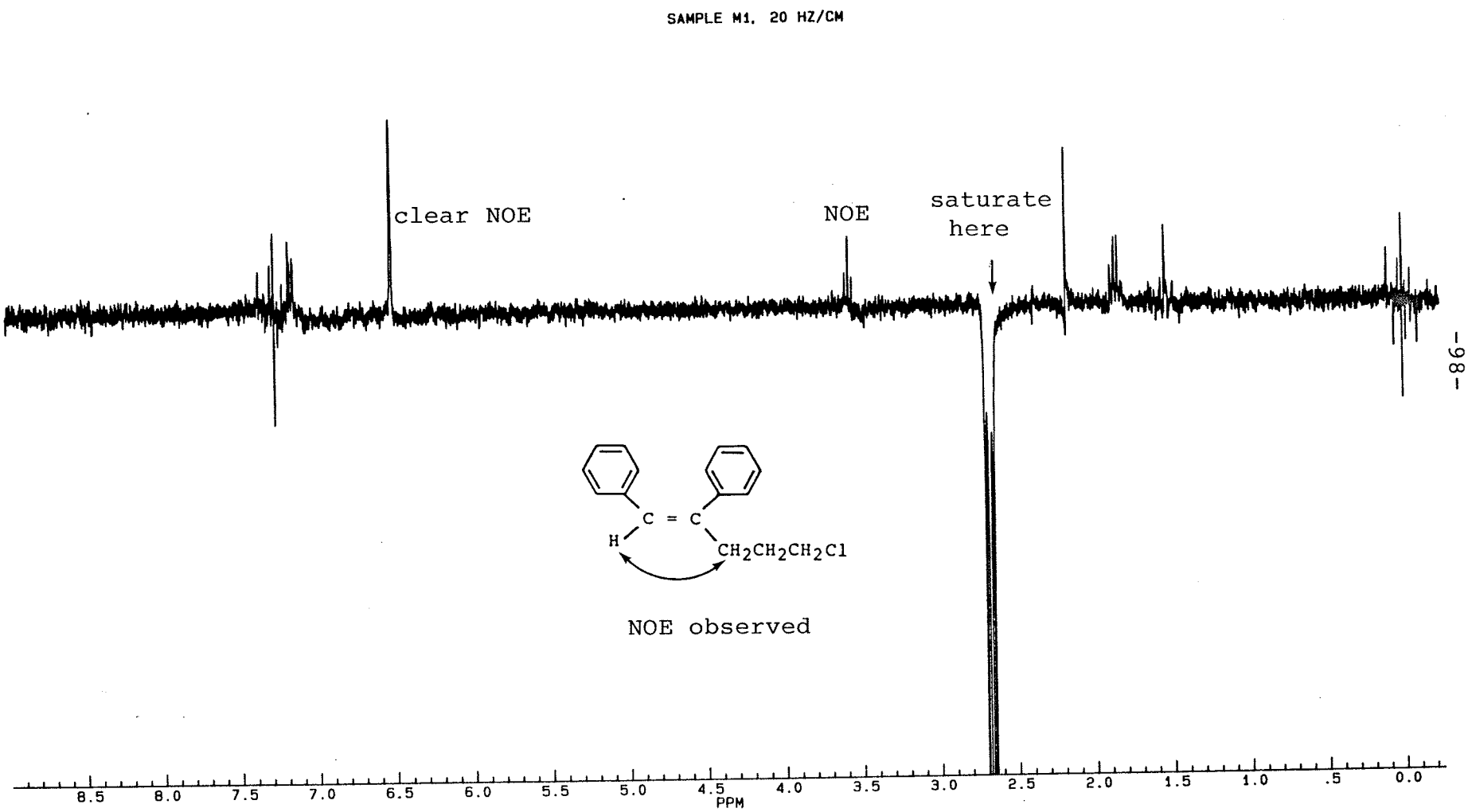


Fig. 11 Z-5-Chloro-1,2-diphenylpent-1-ene (4b).

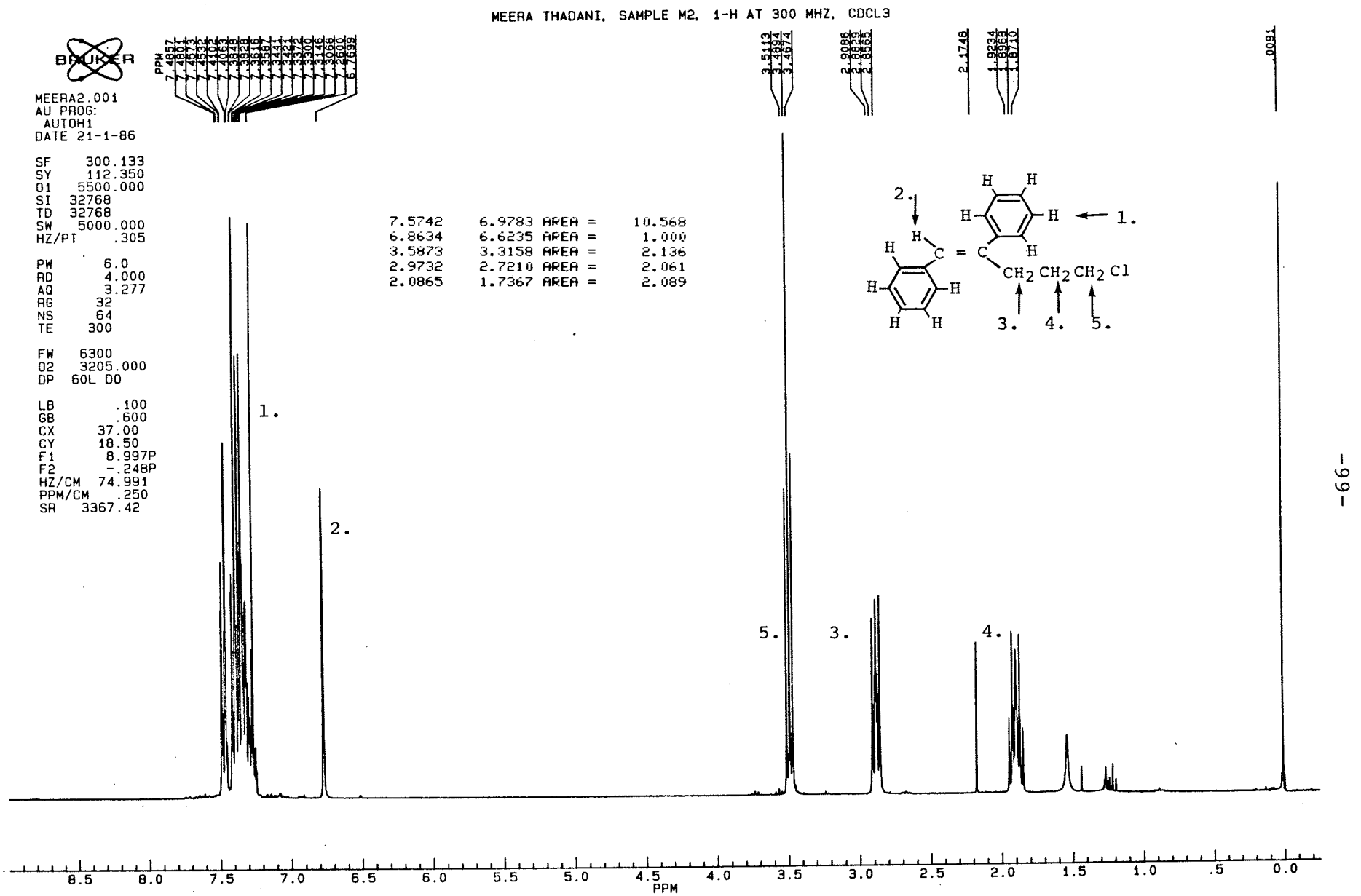


Fig. 12 E-5-Chloro-1,2-diphenylpent-1-ene (4a).

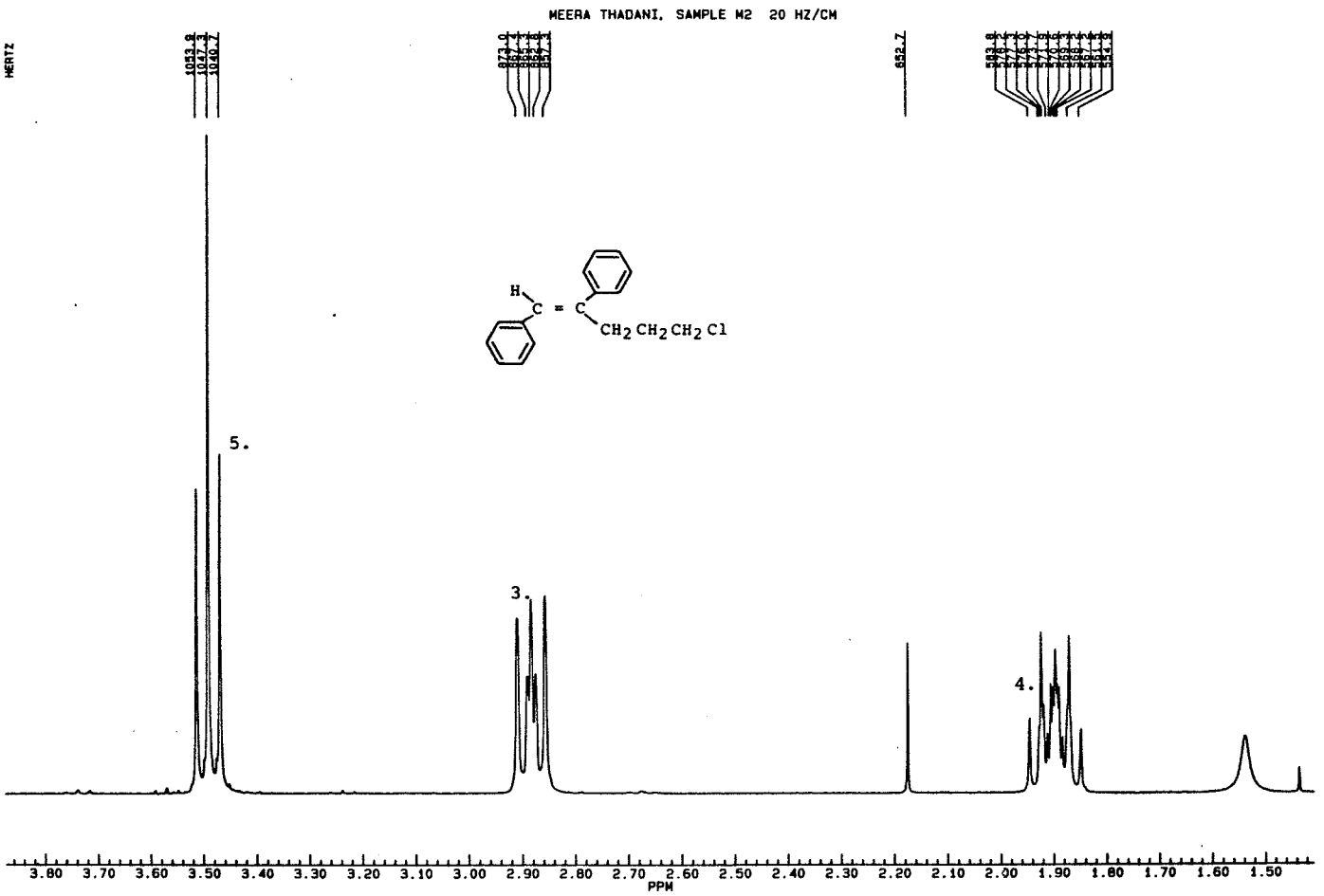


Fig. 13 E-5-Chloro-1,2-diphenylpent-1-ene (4a).

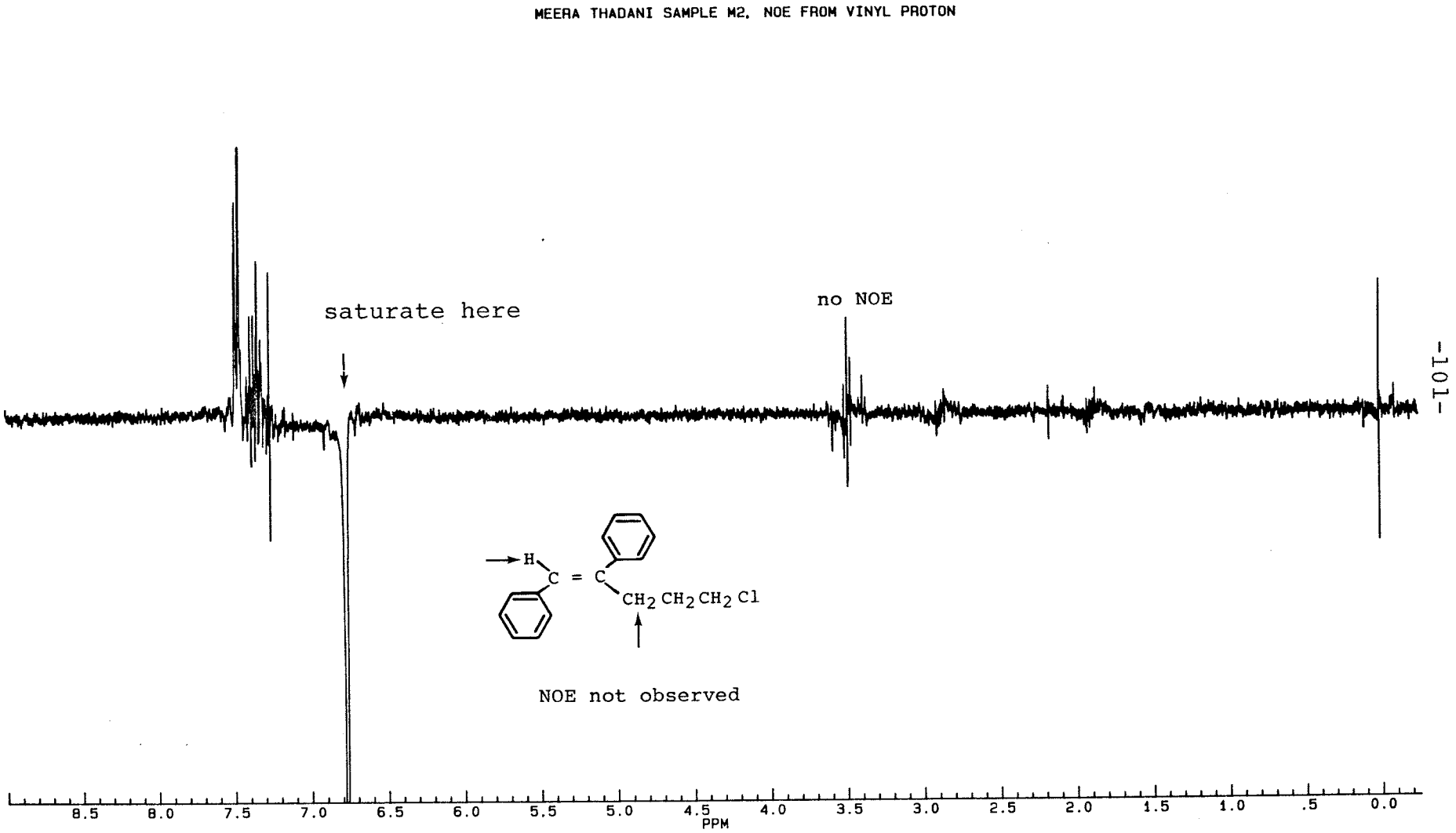


Fig. 14 E-5-Chloro-1,2-diphenylpent-1-ene (4a).

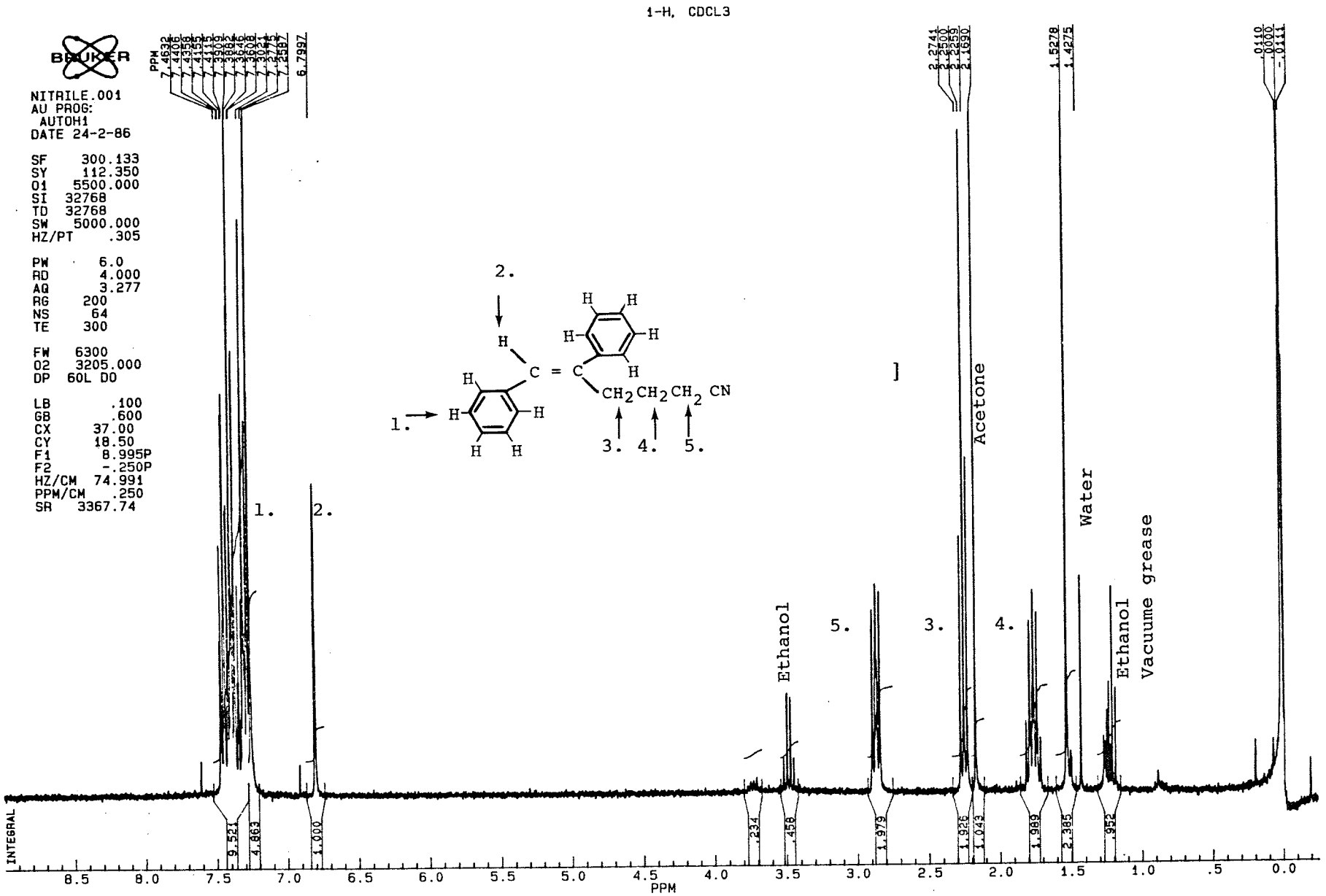


Fig. 15 E-5-Cyano-1,2-diphenylpent-1-ene (5).

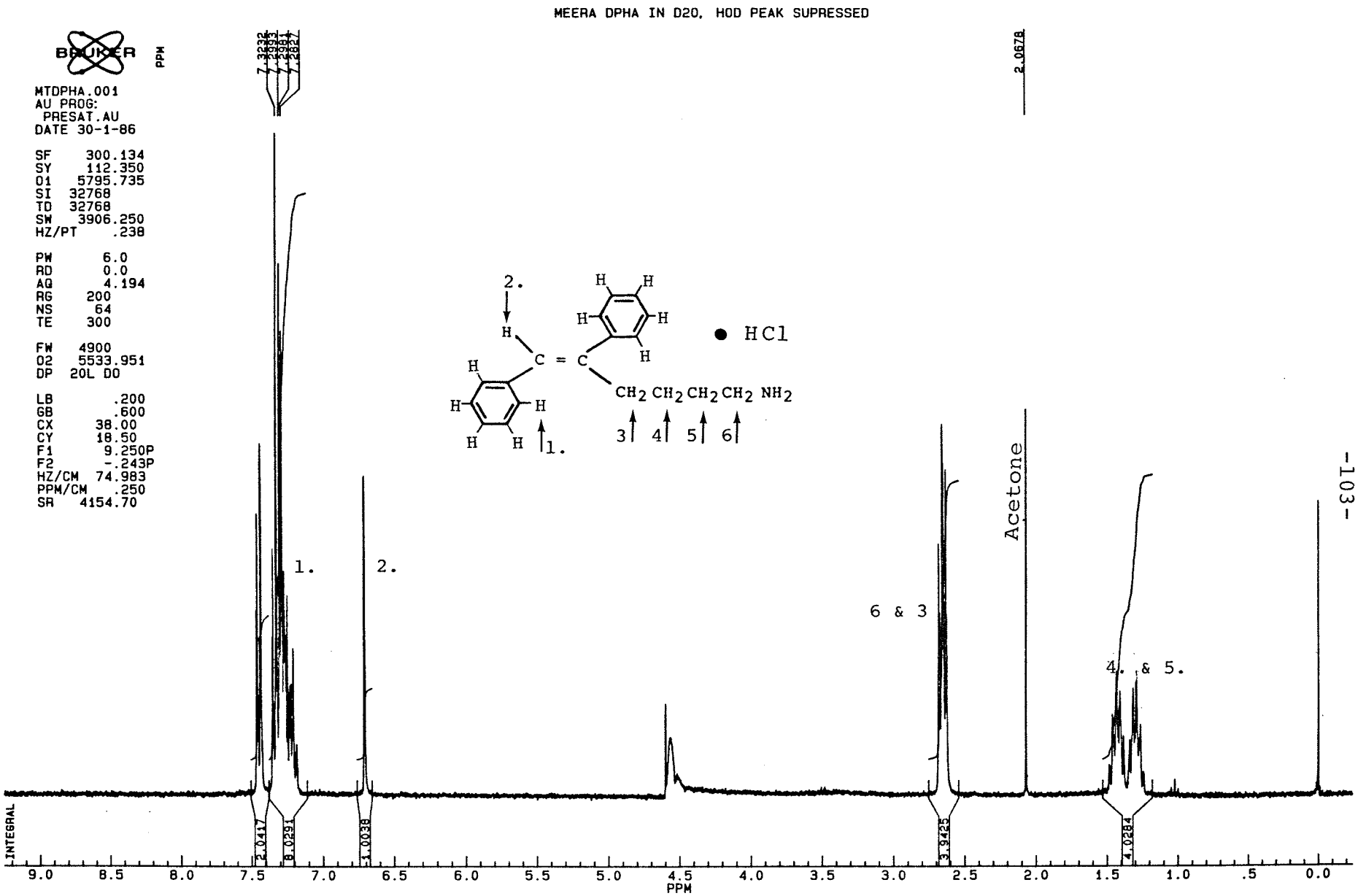


Fig. 16 E-6-Amino-1,2-diphenylhex-1-ene hydrochloride (6).

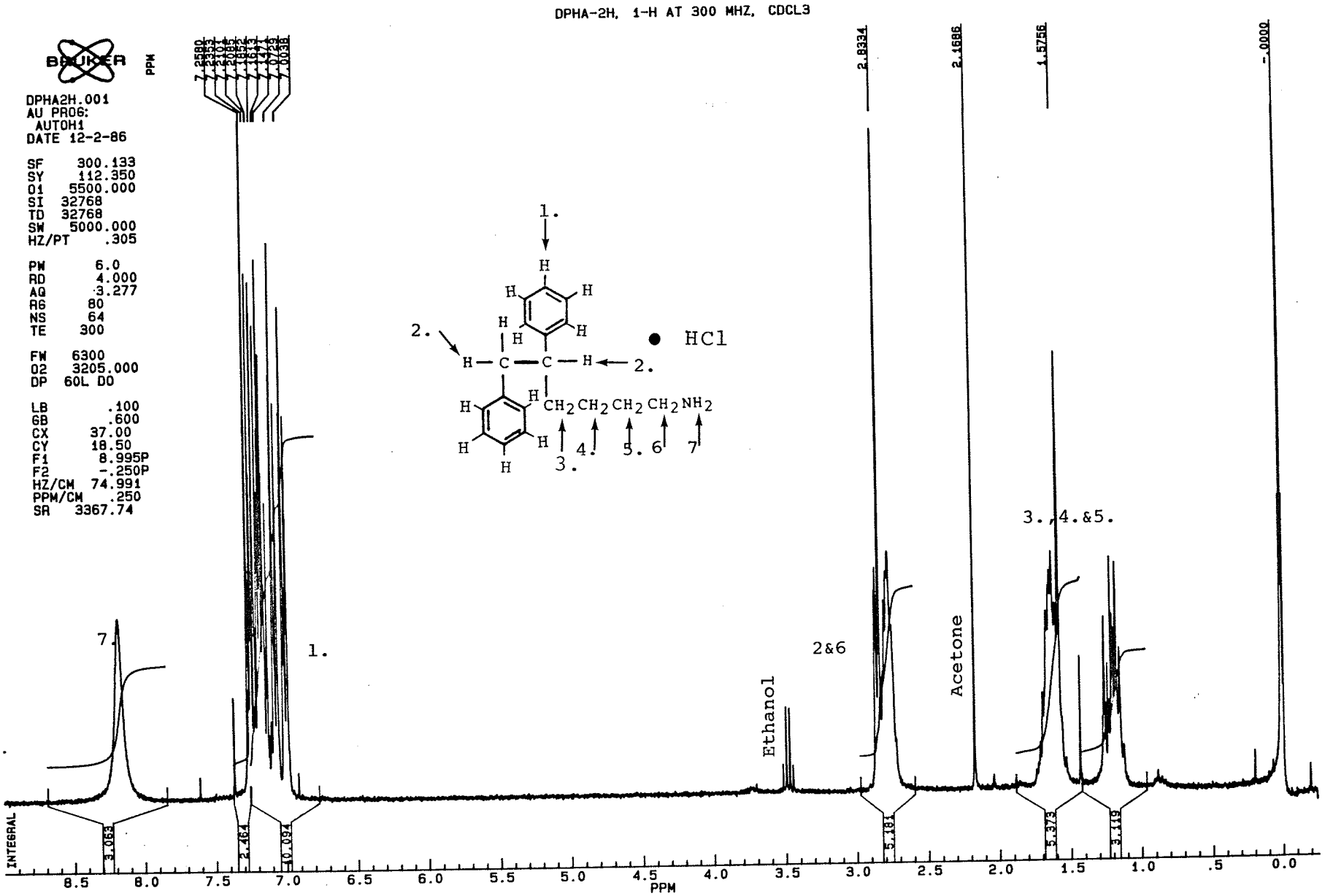


Fig. 17 6-Amino-1,2-diphenylhexane hydrochloride (7).

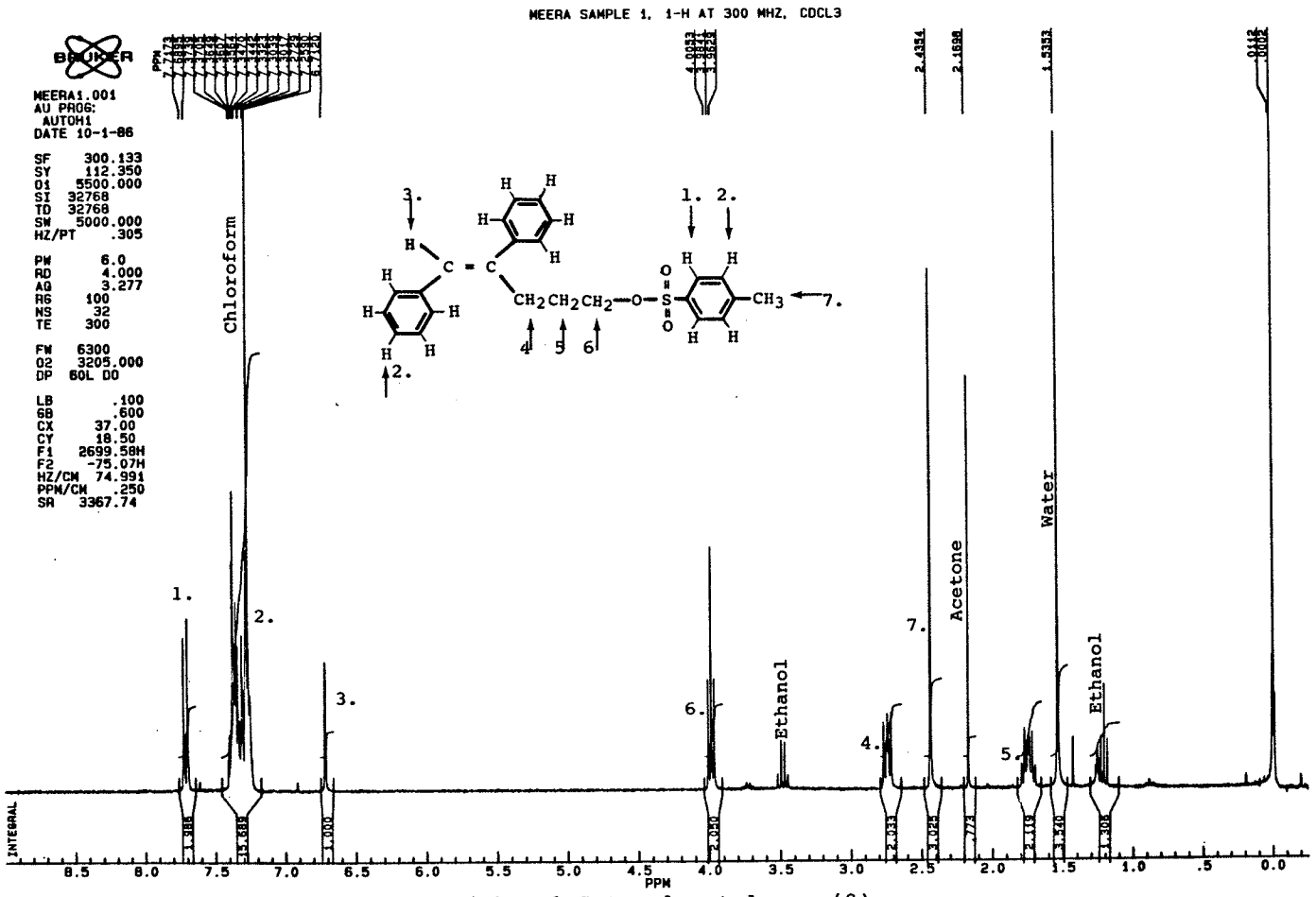


Fig. 18 E-1,2-Diphenyl-5-tosylpent-1-ene (8).

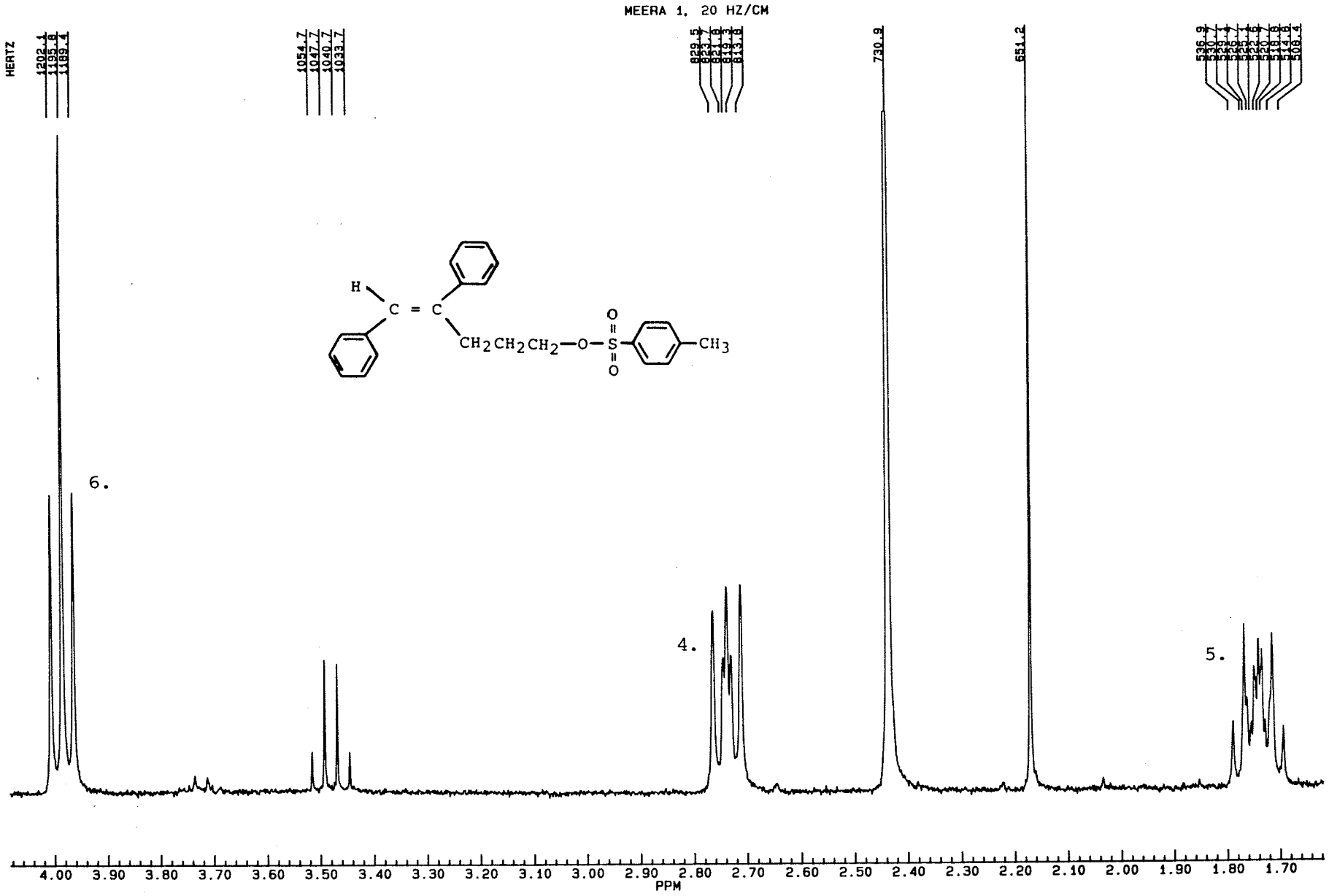


Fig. 19 E-1,2-Diphenyl-5-tosylpent-1-ene (8).

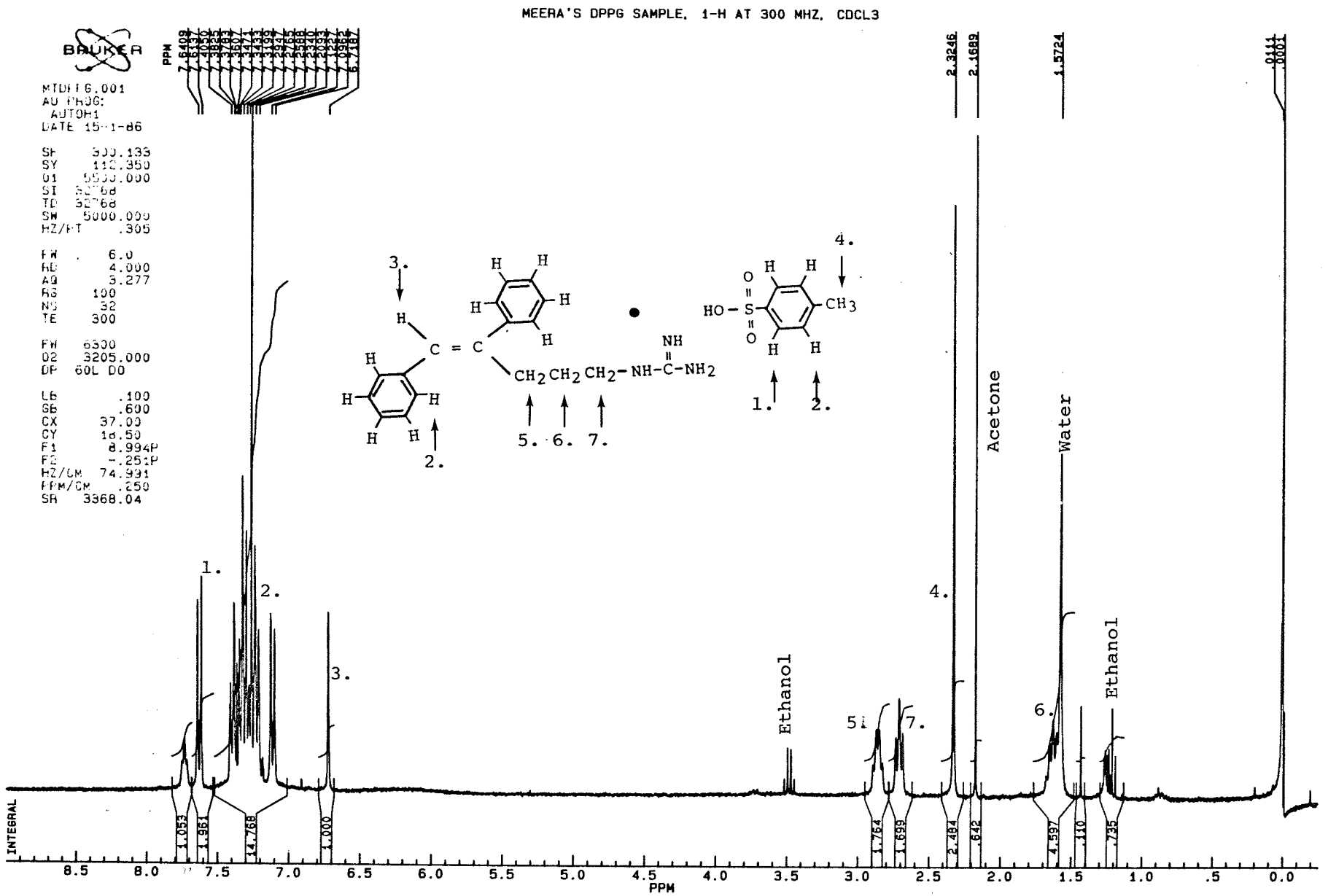


Fig. 20 E-1,2-Diphenyl-5-guanidinopent-1-ene tosylate (9).

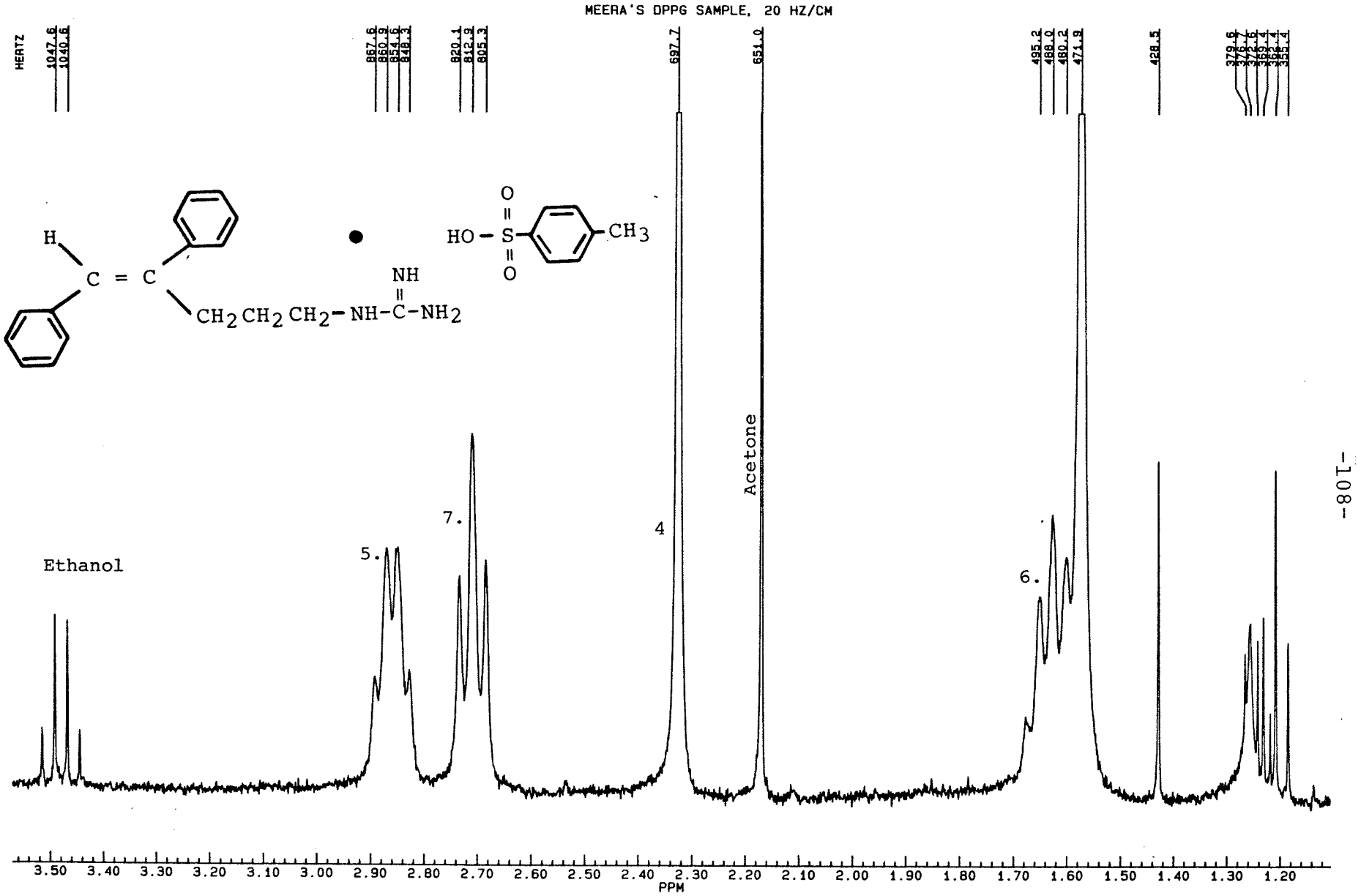


Fig. 21 E-1,2-Diphenyl-5-guanidinopent-1-ene tosylate (9).

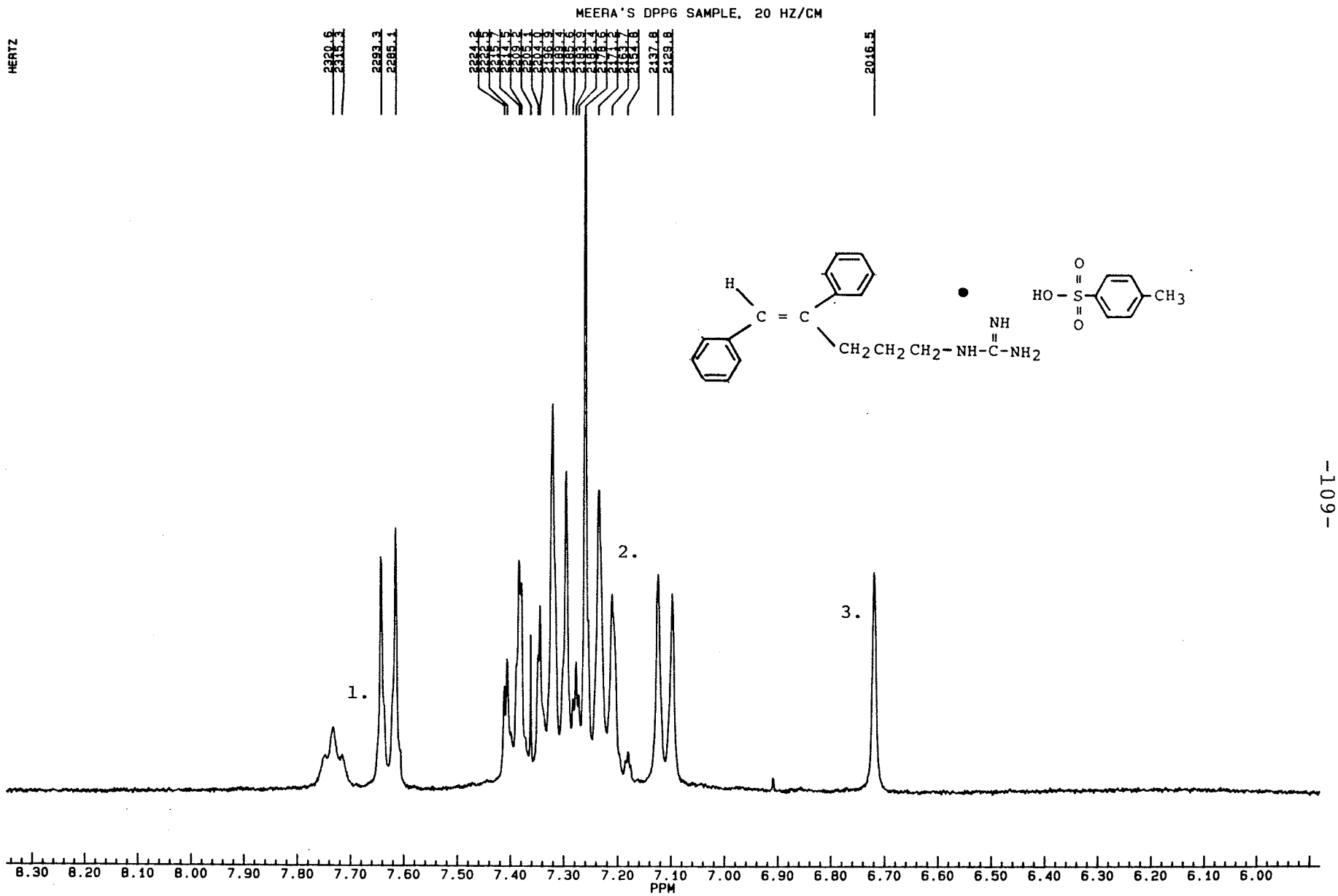


Fig. 22 E-1,2-Diphenyl-5-guanidinopent-1-ene tosylate (9).

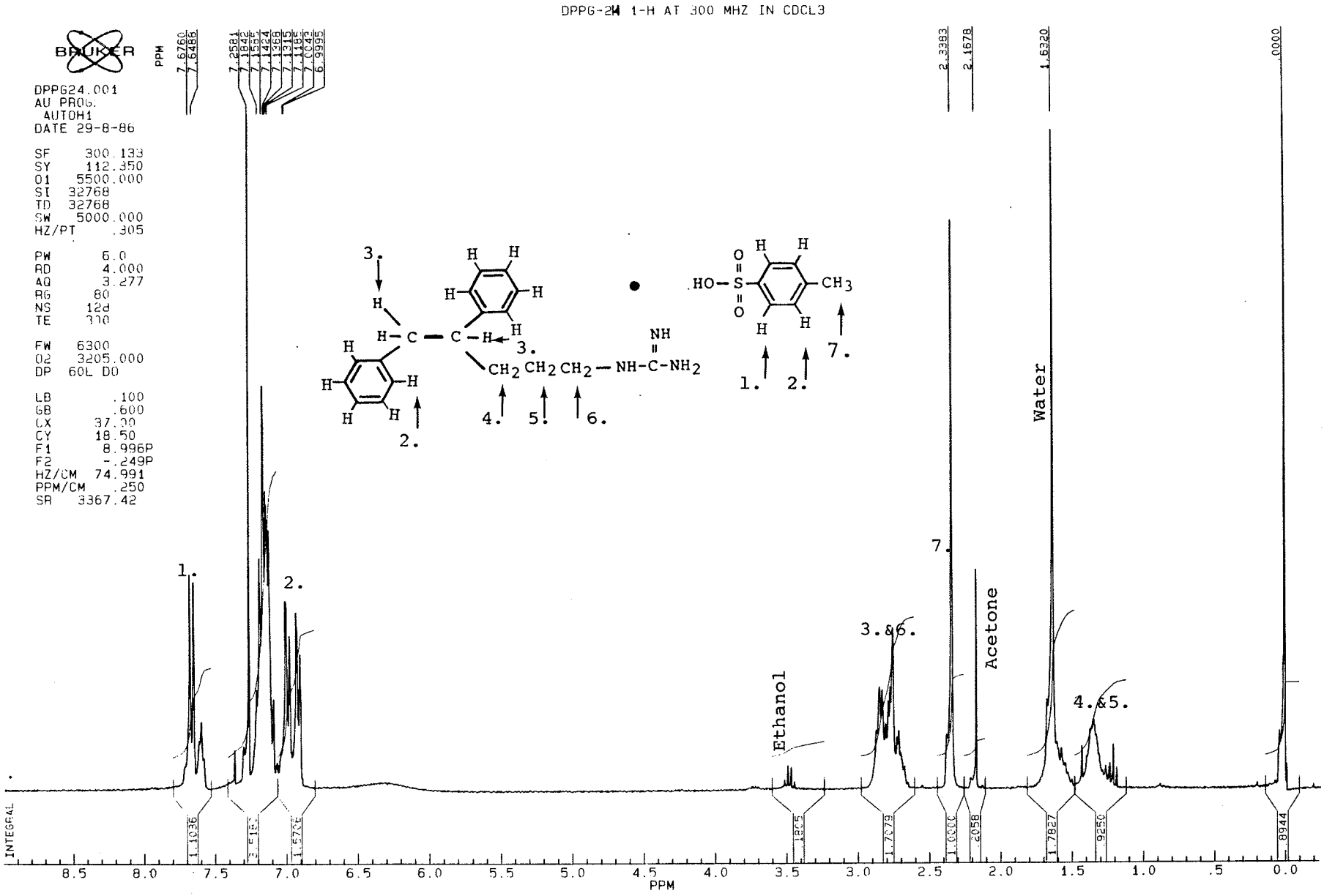


Fig. 23 1,2-Diphenyl-5-guanidinopentane tosylate (10).

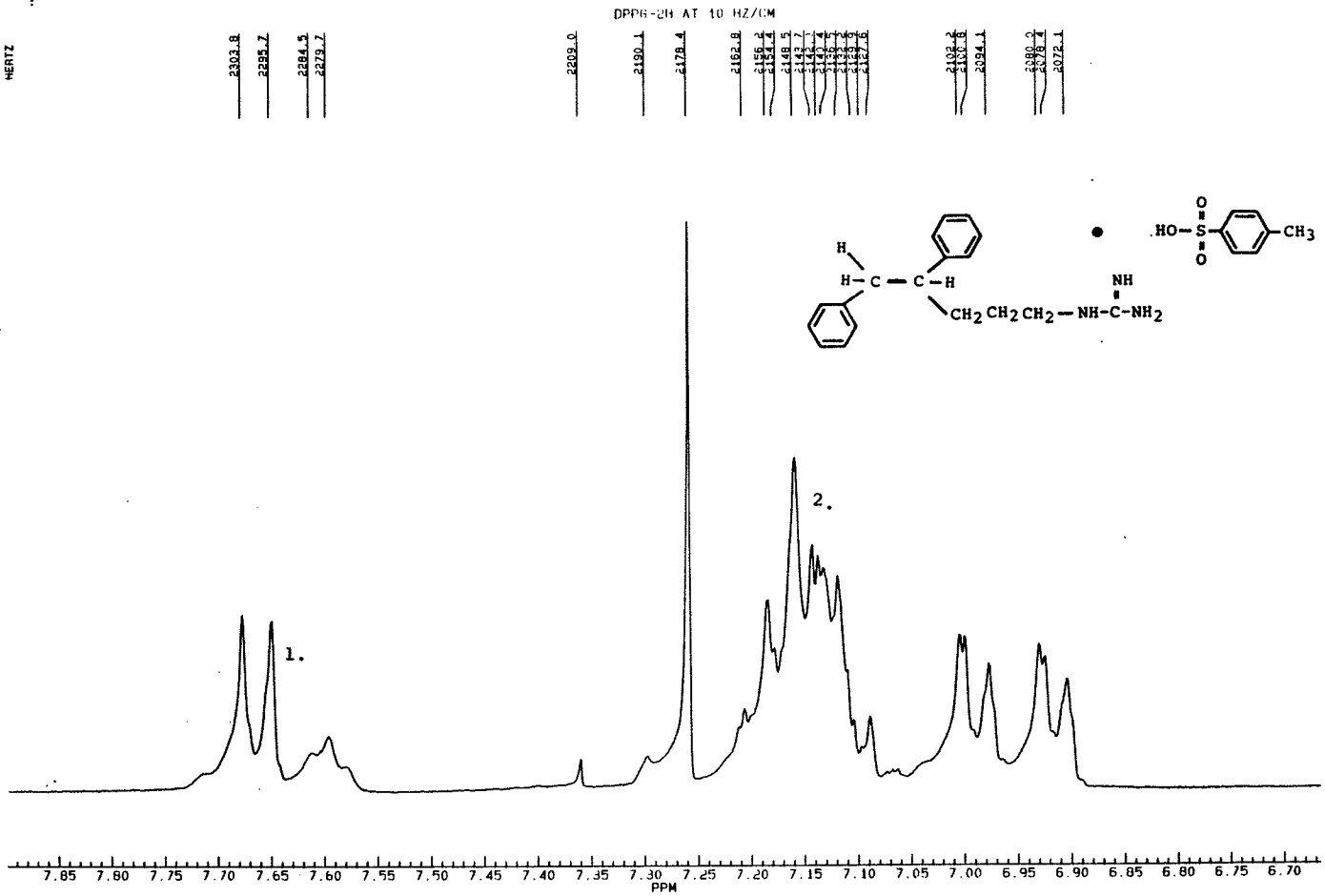


Fig. 24 1,2-Diphenyl-5-guanidinopentane tosylate (10).

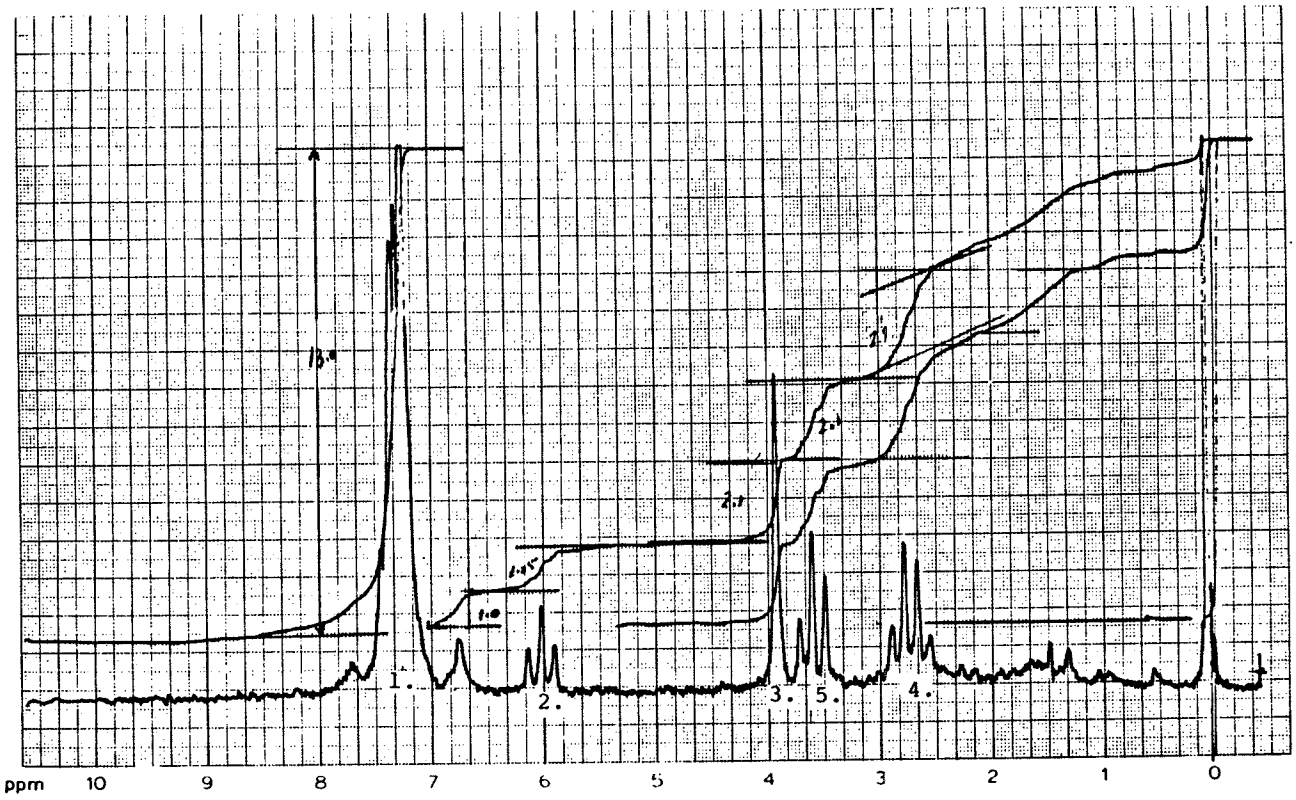


Fig. 25 NMR spectrum of 5-chloro-1,2-diphenylpent-2-ene (Varian 60 mHz) E/Z mixture

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